Liliana Raquel Leite Martins

Antimicrobial resistance analysis of fecal *Escherichia coli* and *Enterococcus* spp. isolates from dogs and cats: prevalence, assessment of potential risk factors and ability of multidrug-resistant strains to spread within household

Tese de Candidatura ao grau de Doutor em Ciências Veterinárias submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador - Professor Doutor Paulo Manuel Rodrigues Martins da Costa
Categoria - Professor Auxiliar
Afiliação - Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto (UP)

Co-orientador - Professor Doutor Augusto José Ferreira de Matos
Categoria - Professor Auxiliar
Afiliação - Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto (UP)

Aos meus filhos

“O médico que só sabe Medicina, nem Medicina sabe”

Abel de Lima Salazar

Publicações

Em obediência ao disposto no n.º 1 do Artigo 34º do Decreto-Lei n.º 74/2006, publicado em Diário da República, 1ª série, nº 60 de 24 de Março de 2006, e republicado pelo Decreto-Lei nº 115/2013, publicado em Diário da República, 1ª série, nº 151 de 07 de Agosto de 2013, que procede à terceira alteração do Decreto-Lei nº 74/2006, de 24 de Março de 2006, a autora declara que participou na conceção e na execução do trabalho experimental, bem como na interpretação dos resultados e na redação dos trabalhos, publicados e em publicação, que fazem parte integrante desta tese e/ou que dela advêm.

Artigos que fazem parte integrante da Tese

I. Martins, L.R., Pina, S.M., Simões, R.L., de Matos, A.J., Rodrigues, P., da Costa, P.M. (2013). Common phenotypic and genotypic antimicrobial resistance patterns found in a case study of multiresistant *E. coli* from cohabitant pets, humans, and household surfaces. *Journal of Environmental Health*. 75(6):74-81.

II. Leite-Martins, L., Meireles, D., Bessa, L.J., Mendes, A., de Matos, A.J., Martins da Costa, P. (2014). Spread of Multidrug-Resistant *Enterococcus faecalis* Within the Household Setting. *Microbial Drug Resistance*. March 11. (*Epub ahead of print*).

III. Leite-Martins, L., Meireles, D., Beça, N., Bessa, L.J., de Matos, A.J.F., da Costa, P.M. (2014). Spread of multidrug-resistant *Escherichia coli* within Domestic Aggregates (humans, pets and household environment). *Microbes and Environments* (*Submitted*).

IV. Leite-Martins, L., Mahú, M.I., Costa, A.L., Mendes, A., Lopes, E., Mendonça, D.M.V., Niza-Ribeiro, J., de Matos, A.J.F., da Costa, P.M. (2014). Prevalence of antimicrobial resistance in enteric *Escherichia coli* from domestic pets and assessment of associated risk markers using a Generalized Linear Mixed Model. *Preventive Veterinary Medicine* (*Submitted*).

V. Leite-Martins, L., Mahú, M.I., Costa, A.L., Vaz-Pires, P., Niza-Ribeiro, J., de Matos, A.J.F., da Costa, P.M. (2014). Prevalence of antimicrobial resistance in enteric *Enterococcus* spp. from domestic pets and assessment of associated risk factors using a Generalized Linear Mixed Model. *Journal of Medical Microbiology* (*Submitted*).

Outros trabalhos científicos motivados pela presente Tese

Artigo em revista de circulação internacional com arbitragem científica

I. Beça, N.M., Bessa, L., Mendes, A., Santos, J., Leite-Martins, L., Matos, A., Martins da Costa, P. (2014). Coagulase-positive *Staphylococcus* - prevalence and antimicrobial resistance in companion animals, veterinary professionals and clinical environment. *Journal of the American Animal Hospital Association*. (*In press*).

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II. Martins da Costa, P., Leite-Martins, L., Antunes, F., Simões, R. (2010). Transferência de bactérias resistentes aos antimicrobianos entre nichos ecológicos interligados: homem, animais e ambiente. *Revista da Faculdade de Medicina de Lisboa*, série III 15 (5/6): 319-326.

Publicações em atas de encontros científicos

Comunicações Orais

III. Leite-Martins, L. (2013). Prevalência da resistência aos antimicrobianos em *Escherichia coli* e *enterococcus* spp. isolados em cães e gatos e estudo dos respetivos fatores de risco. *VIII Congresso OMV*. Lisboa, Portugal. 30 de Novembro a 01 de Dezembro de 2013.

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V. Meireles, D.M., Martins, L.R., Bessa, L.J., Mendes, Â.J., Cunha, S.A., Matos, A., da Costa, P.M. (2014). Estudo da partilha de clones bacterianos entre animais de companhia, coabitantes humanos e superfícies domésticas. *VI Congresso da Sociedade Portuguesa de Ciências Veterinárias*: Praxis e futuro, Oeiras, Portugal, 3-5 de Abril. (Pp.127).

VI. Leite-Martins, L**.**, Beça, N., Lopes, E., Frias, C., Matos, A., Martins da Costa, P. (2012). In-home and through-home transmission of antimicrobial resistance between human and pets. *II International Conference on Antimicrobial Research – ICAR 2012*, Lisbon, Portugal, 21-23 November. (Pp:410).

VII. Beça, N.M., Simões, R.L., Santos, J.C., Lopes, E., Leite-Martins, L., Matos, A., Martins da Costa, P. (2012). Culture media isolation of *Staphylococcus pseudointermedius* and *Staphylococcus* spp. coagulase positive prevalence in domestic animals, Veterinary practitioners, Veterinary auxiliary workers and environment of a Veterinary hospital. *II International Conference on Antimicrobial Research – ICAR 2012*, Lisbon, Portugal, 21-23 November. (Pp:387).

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ABBREVATIONS

AMC	Amoxicillin-clavulanic acid
AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
ATM	Aztreonam
AZM	Azithromycin
CAMV	Centro de atendimento Médico-Veterinário
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
CEF	Cephalothin
CHL	Chloramphenicol
CIP	Ciprofloxacin
CTX	Cefotaxime
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Program
DGAV	Direção-Geral de Alimentação e Veterinária
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
e.g.	*exempli gratia*
EAAD	European Antibiotic Awareness Day
ERI	Erythromycin
ESBL	Extended-Spectrum Beta-Lactamases
et al.	*et alii*
ExPEC	Extra-intestinal Pathogenic *E. coli*
FAO	Food and Agriculture Organization
Fig.	Figure
FOX	Cephoxitin
GEN	Gentamicin
GSP	Good Stewardship Practice
HGT	Horizontal Gene Transfer
IPM	Imipenem
KAN	Kanamycin

NAL	Nalidixic acid
NARMS	National Antimicrobial Resistance Monitoring System
NIT	Nitrofurantoin
OMV	Ordem dos Médicos Veterinários
QD	Quinupristin/dalfopristin
RIF	Rifampicin
SCOPE	Surveillance and Control of Pathogens of Epidemiologic Importance
SENTRY	Antimicrobial surveillance Program
STR	Streptomycin
SVARM	Swedish Veterinary Antimicrobial Resistance Monitoring Program
SWEDRES	Antibiotic Consumption and Resistance in Sweden
SXT	Trimethoprim-sulfamethoxazol
TEC	Teicoplanin
TET	Tetracycline
TOB	Tobramycin
UK	United Kingdom
UPVet	Clínica Veterinária de Animais de Companhia do ICBAS / UP
USA	United States of America
UTI	Urinary Tract Infection
VAN	Vancomycin
VRE	Vancomycin Resistant *Enterococcus*

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RESUMO

A resistência aos antimicrobianos (AMR) é atualmente um dos principais problemas de saúde pública a nível mundial. Sem que se vislumbrem medidas corretivas imediatas, a conjugação da emergência de bactérias multirresistentes com o enfraquecimento do interesse da indústria farmacêutica na descoberta de novos compostos antimicrobianos invoca o espetro de estarmos a progredir em direção a uma era pós-antimicrobiana, que nos deixará indefesos mesmo perante as infeções bacterianas mais vulgares. A emergência e a disseminação massiva dos determinantes de resistência é resultado de décadas de uso de antibióticos, no homem e nos animais, sem um conhecimento cabal do impacto ecológico destes compostos na flora bacteriana. A evolução da medicina veterinária e a sensibilização da população para a saúde e bem-estar animais conduziram a um incremento quer da longevidade dos animais de companhia, quer da frequência de patologias crónicas e imunodebilitantes, amplamente associadas a maior probabilidade de carecerem de tratamentos antimicrobianos que, por sua vez, promoveram a emergência de AMR nestes animais. Para defesa da saúde humana e animal, é importante recolher informação epidemiológica, relativa a cães e gatos, que auxilie a antibioterapia empírica e que, ao mesmo tempo, apoie o desenvolvimento de estratégias conservativas para o controlo dos riscos de transmissão de estirpes multirresistentes entre animais de companhia e os seus coabitantes humanos.

Considerando as referidas preocupações, dois objetivos foram propostos para o presente estudo: i) a monitorização dos perfis de AMR de *Escherichia coli* e *Enterococcus* spp. isolados em fezes de cães e gatos atendidos na Clínica Veterinária da Universidade do Porto (UPVet), Portugal, e estudo dos respetivos fatores de risco; e ii) a avaliação da disseminação e partilha de bactérias ou de determinantes genéticos de resistência antimicrobiana através do ambiente doméstico, considerando coabitantes humanos, animais de companhia e superfícies e objetos frequentemente tocados por ambos.

Para o trabalho de monitorização recolheram-se zaragatoas rectais em 81 cães e 30 gatos que não haviam sido submetidos a qualquer tratamento antibioterapêutico nos quatro meses que antecederam a colheita. A seleção dos animais foi efetuada por um método sistemático aleatório, entre Setembro de 2009 e Maio de 2012. Os proprietários assinaram um termo de consentimento, preencheram um questionário e permitiram a amostragem dos animais, através de zaragatoa rectal, para posterior isolamento de *E. coli* e enterococos. A Comissão de Ética do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto deu a sua aprovação prévia à realização do estudo.

Obtiveram-se 396 isolados de *E. coli* e 315 isolados de *Enterococcus* spp. Uma proporção considerável de isolados de *E. coli* revelou resistência à ampicilina (51,3%), à cefalotina (46,7%), à tetraciclina (45,2%) e à estreptomicina (43,4%). Os enterococos mostraram-se mais resistentes à tetraciclina (67,0%), à rifampicina (60,3%), ao aztreonam (58,4%), à quinupristina/dalfopristina (54,0%) e à eritromicina (53,0%). Não se encontraram resistências à nitrofurantoína nem ao imipenem. O "tratamento prévio com quinolonas" foi considerado o principal fator de risco para a presença de AMR em 12 (ampicilina, cefalotina, ceftazidima, cefotaxima, ácido nalidíxico, ciprofloxacina, gentamicina, tetraciclina, estreptomicina, cloramfenicol, trimetoprim-sulfametoxazol e aztreonam) dos 15 antimicrobianos testados para *E. coli* e em 3 (cloranfenicol, ciprofloxacina e azitromicina) dos 9 antimicrobianos testados para enterococos. Os "hábitos de coprofagia" foram também positivamente associados a um maior risco de AMR para *E. coli* (ampicilina, amoxicilina-ácido clavulânico, cefamicina, ciprofloxacina, estreptomicina e trimetoprim-sulfametoxazol) e para os enterococos, relativamente à tetraciclina, rifampicina, gentamicina, cloranfenicol, ciprofloxacina, eritromicina e azitromicina.

Em função dos perfis de resistência antimicrobiana encontrados e/ou historial antibioterapêutico dos animais, alguns proprietários foram abordados no sentido de colaborarem na segunda fase do estudo, para se proceder à recolha de amostras nos coabitantes humanos e animais, assim como em algumas superfícies e objetos de uso frequente no quotidiano doméstico. Realizaram-se três estudos para avaliação da potencial disseminação de enterococos em agregados domésticos, originários de dois cães e um gato amostrados para o estudo de prevalência; para os trabalhos com *E. coli* participaram três agregados selecionados a partir do universo de 81 cães amostrados. Os resultados obtidos evidenciaram a disseminação de *E. coli* e *Enterococcus faecalis* multirresistentes entre animais de companhia (cães e gatos) e respetivos proprietários. As mesmas estirpes foram também encontradas disseminadas em diversos objetos e superfícies do ambiente doméstico.

Os resultados do presente estudo deveriam alertar a classe médico-veterinária para o problema da emergência da AMR nos animais de companhia, para os fatores de risco que a regulam, assim como para a possibilidade de disseminação intra- e inter-espécies.

ABSTRACT

Antimicrobial resistance (AMR) is currently a major threat to public health around the world. In the absence of urgent corrective and protective actions, the worrying conjuncture of bacteria developing resistance against all known classes of antibiotics at a time that pharmaceutical industry was weakening investment in discovering new ones, mankind is heading towards a post-antibiotic era, in which many common bacterial infections will no longer have a cure. The increasing emergence and spread of AMR is the result of decades of usage of antibiotics in humans and animals with a misperception of the ecological impact of this usage on the bacterial flora. Advances in veterinary medicine and heightened sensibility of population towards the health and welfare of pets conducted to a rise in pets' longevity with a substantial augment in chronic debilitating and immunocompromising conditions and higher probability for needing antimicrobial treatments, guiding to the emergence of AMR amongst these animals. Due to both animal and human health concerns, investigation efforts involving dogs and cats are needed to provide epidemiological information that could guide antimicrobial empiric therapy and help the development of conservative risk management strategies to mitigate the transmission of multidrug-resistant strains between them and their human cohabitants.

Bearing in mind the above concerns, two main purposes were addressed for the present work: i) a survey study of AMR profiles of fecal *Escherichia coli* and *Enterococcus* spp. from dogs and cats attending the Small Animal Veterinary Clinic of Porto University (UPVet) in Portugal, with an estimation of the respective risk factors; and ii) the assessment of within household spread and share of antimicrobial resistant determinants or bacteria, taking into consideration cohabitant humans and pets and common touched objects and surfaces.

For the surveillance work, fecal samples were obtained from 81 dogs and 30 cats that were not submitted to any antimicrobial therapy within the preceding four months. A random systematic approach was adopted to select the animals for the survey study at the UPVet, from September 2009 to May 2012. The owners were asked to sign in a term of acceptance, to fill a questionnaire and to allow the collection of fecal samples from their pets using rectal swabs in order to perform *E. coli* and enterococci isolation. A previous approval was obtained from the Ethics Committee of the Abel Salazar Institute for the Biomedical Sciences, University of Porto.

Three hundred and ninety six *E. coli* and 315 enterococci isolates were obtained. A considerable proportion of *E. coli* isolates displayed resistance to ampicillin (51.3%), cephalothin (46.7%), tetracycline (45.2%) and streptomycin (43.4%). Enterococci were more resistant to tetracycline (67.0%), rifampicin (60.3%), aztreonam (58.4%), quinupristin/dalfopristin (54.0%) and erythromycin (53.0%). No resistances were found to nitrofurantoin and imipenem. It was found that “Previous quinolone treatment” was the main risk factor for the presence of AMR in 12 (ampicillin, cephalothin, ceftazidime, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, tetracycline, streptomycin, chloramphenicol, trimethoprim-sulfamethoxazol and aztreonam) out of the 15 antimicrobials assessed for *E. coli* and in 3 (chloramphenicol, ciprofloxacin and azithromycin) out of the 9 of the antimicrobials assessed for enterococci. “Coprophagic habits” were also positively associated with an increased risk of AMR in *E. coli* (for ampicillin, amoxicillin-clavulanic acid, cephamycin, ciprofloxacin, streptomycin, and trimethoprim-sulfamethoxazol) and in enterococci (for tetracycline, rifampicin, gentamicin, chloramphenicol, ciprofloxacin, erythromycin and azithromycin).

Considering the resistance profiles found into some enteric bacteria and/or the previous clinical records of the animals, some of the owners were asked to enter the second branch of the study, expanding the investigation to the humans and pets cohabitants as well as to some frequently touched household objects and surfaces. Domestic aggregates from two dogs and one cat agreed to collaborate in the enterococci spread investigation whereas three dog owners’ endorsed the *E. coli* dissemination study. Results showed that multidrug-resistant *E. coli* and *Enterococcus faecalis* can happen between pets (dogs and cats) and owners. Those strains were also disseminated throughout home and household objects and surfaces.

Results from the present study should alert veterinarians for the AMR emergence problem in small animals, the risk factors that regulate it as well as of its ways of intra- and inter-species spread.

Chapter 1

GENERAL INTRODUCTION

RATIONALE AND AIMS

1.1. GENERAL INTRODUCTION

1.1.1. The phenomenon of antimicrobial resistance

Antibiotics are one of the most important therapeutic discoveries in medical history. When antibiotics were first introduced in the 1940s, they were called “wonder drugs”, the “miracle” of modern medicine (WHO, 2011). Major diseases that killed millions of people could then be treated. Its widespread use for over 70 years, however, “educated” bacteria to become resistant and, apparently, the global resistance phenomenon has caught everyone unprepared (Prescott, 2014). According to the World Health Organization (WHO, 2012a), the world is heading towards a post-antibiotic era, in which many common infections will no longer be cured with antibiotics because bacteria are becoming largely resistant to them (Andersson and Hughes, 2010; EAAD, 2013). The increasing global resistance rates in many bacterial species, responsible for both community- and hospital-related infections (*Enterobacteriaceae*, staphylococci and enterococci,), as well as the emergence and rapid dissemination of new mechanisms of resistance (*e.g.* extended-spectrum beta-lactamases (ESBL) and carbapenemases), are two staggering phenomena (Carlet *et al*., 2012). Infections by resistant bacteria are currently quite common, and some pathogens are resistant to multiple types or classes of antibiotics (CDC, 2013). Portugal is not immune to this problem, with alarming detection rates of ESBL producing and fluoroquinolone-resistant *E. coli* isolates in both nosocomial and community infections (Machado *et al.*, 2006; Mendonça *et al.,* 2007; Guimarães *et al.,* 2009). Resistance dramatically reduces first-line and second-line antibiotic treatment options, forcing healthcare providers to use antibiotics that may be more toxic to the patient, more expensive and frequently less effective, thus increasing the risk of complications, delayed recuperation, long-term disability and even fatal outcomes (Andersson and Hughes, 2010; Carlet *et al*., 2012; CDC, 2013). Additionally, the increasing resistance to last-line antibiotics, such as carbapenems used to treat healthcare-associated infections, means that presently carbapenem-resistant infections are being treated with old and toxic drugs, which may be considered a drawback in antimicrobial therapy (EAAD, 2013).

The implications of AMR emergence go beyond the resurgence of deadly infections; it will also threat many life-saving and life-prolonging interventions attending to the emergence of highly-resistant pathogens in hospital settings (Bassetti and Righi, 2013; EAAD, 2013).

To address these issues, it is imperative that novel classes of antibiotics demonstrating activity against bacterial strains resistant to the existing ones are introduced into the clinical practice (Georgopapadakou, 2013). Nonetheless, only a small number are currently in development and most belong to the existing classes: lipoglycopeptides, cephalosporins, amino-glycosides, ketolides, oxazolidinones and antifolates (Projan and Bradford, 2007). Worryingly, antibiotics under development target almost exclusively Gram-positive bacteria (O'Neill, 2008). There is thus an urgent need for compounds active against Gram-negative bacteria, particularly *Enterobacteriaceae* displaying resistance against currently available drugs (Bassetti and Righi, 2013).

The threatening hospital-emerging "superbugs" are just the extreme expression of a much broader and disturbing phenomenon. The development of resistance is a natural biological process that will occur, sooner or later, for every drug. It is based on the genetic plasticity of bacteria and has emerged as the consequence of a "selective pressure" exerted by the antimicrobial usage in human and veterinary medicine, animal and fish production, agriculture and food technology (van de Sande-Bruinsma *et al*., 2008; da Costa *et al*., 2013). There is considerable evidence that antimicrobial use selects for resistance in commensals and zoonotic pathogens of both humans (Enne, 2010; da Costa *et al.,* 2013; EAAD, 2013) and animals (McEwen and Fedorka-Cray, 2002; Berge *et al*., 2006).

The development of antibiotic resistance is usually associated with genetic changes, either mutations in elements relevant for the activity of the antibiotic, or the acquisition of resistance genes. The later may occur by transduction (mediated by bacteriophages), conjugation (which involves direct cell-to-cell contact and transfer of plasmids or transposons) or transformation, involving the uptake of free DNA that results from bacterial lysis (da Costa *et al*., 2013). Horizontal gene transfer (HGT) among bacteria is crucial for resistance spreading, particularly within mixed bacterial populations such as intestinal microbiota (McDermott *et al.,* 2003; Smillie *et al*., 2011). Co-selection of resistance to more than one antibiotic, owing to genetic linkage of the resistance genes (that can be present in the same plasmid or transposon), is a common feature of resistance acquired by HGT. For that reason, the frequency of resistance to an antibiotic may augment, even if that antibiotic is no longer used (O'Brien, 2002; Summers, 2002; Andersson and Hughes, 2010). In some situations, resistance can be achieved without genetic alterations. These non-inherited resistances are associated to specific phenotypic processes such as growth in biofilms, a stationary growth phase or persistence, swarming motility, and surfactant or flagella synthesis (Kearns, 2010).

In summary, AMR emergence is a natural process that has been vastly accelerated and amplified by several human practices, behaviors and policy failures. Unreasonable and inappropriate use of antimicrobials is by far the major driver of drug resistance (Turnidge and Christiansen, 2005; Enne, 2010; da Costa *et al*., 2013; EAAD, 2013). Thus, it is extremely important to simultaneously adopt numerous interventions or actions in order to restrain or stabilize resistance and gain time while new antibiotics can be developed (Prescott, 2014). Such interventions are based on public health strategies like immunization, infection control, protection of food supplies, antibiotic stewardship, and reduction of person-to-person spread through screening, treatment and education (CDC, 2013). Among those, the ethics of Good Stewardship Practice (GSP) is being highlighted as an active and dynamic process of continuous improvement in antibiotic use that must be approached by all antibiotic users (Weese *et al*., 2013; Prescott, 2014).

The presence of AMR in the commensal microbiota of animals can have a serious impact in human health because these bacteria are most likely to be transferred to humans through i) direct; or ii) indirect contact; iii) the food chain and iv) transference of genetic resistance determinants to zoonotic pathogens (McEwen and Fedorka-Cray, 2002; Guardabassi *et al*., 2004; da Costa *et al*., 2013).

According to Prescott (2014), the complex epidemiology of resistance is such that potentially ‘‘resistance anywhere is resistance everywhere’’. This concept is reflected in Figure 1.

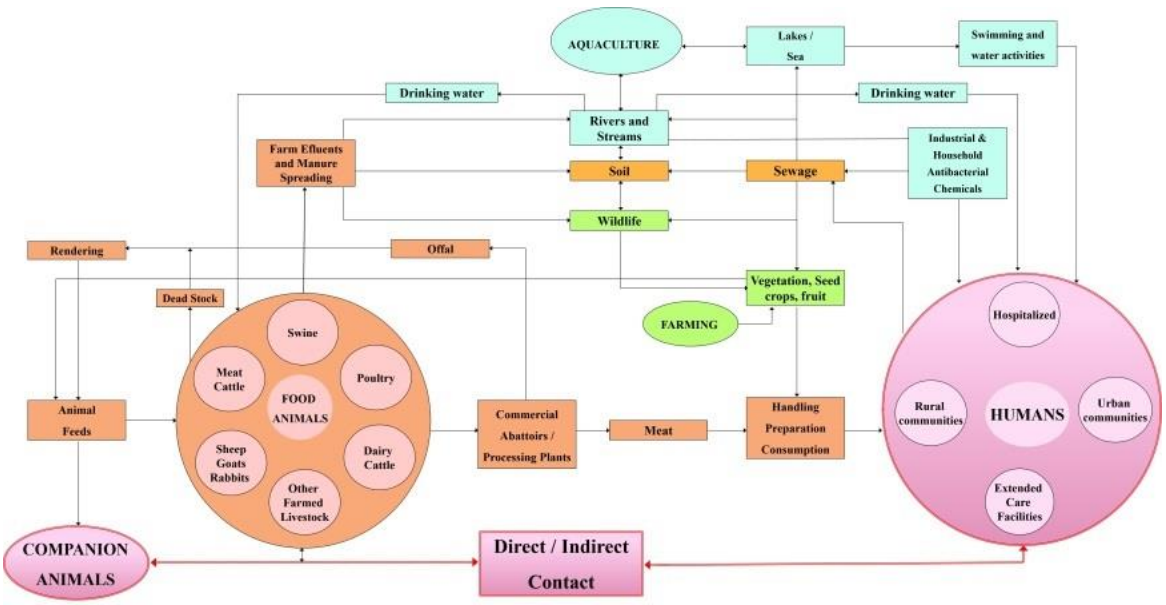

Figure 1. Schematic representation of the global dissemination of antimicrobial resistance (bacteria and resistance genes). Adapted from Prescott (2014).

The food chain is believed to be the most effective way for antimicrobial-resistant bacteria transmission from animals to humans. Relevant data were achieved for *E. coli* (Hammerum *et al*., 2010) as well as for *Enterococcus* spp. (Heuer *et al.*, 2006). Resistant pathogenic or non-pathogenic bacteria are selected in the intestinal flora of animals, contaminate foods of animal origin and colonize or transfer resistance to other bacteria in the human gut (van den Bogaard *et al*., 2000). However, resistant bacteria or their genetic determinants, originated from direct or indirect contact with other sources (*e.g.* contaminated hands, foods, drinks or water) can also achieve and colonize human intestine through the alimentary pathway (Prescott, 2014).

Direct contact is probably the most frequent form for antimicrobial resistant bacteria to pass from animals to humans. Farm workers have frequent contact with skin, feces and urine as well as secretions from oral, nasal or genital cavities of animals. Some reports support the possibility for *E. coli* (or its resistance genes) to be transmitted through direct contact between humans and farm animals such as cattle (Madec *et al*., 2012), pigs (van den Bogaard *et al.*, 2000; Zhao *et al*., 2010), chicken and poultry (Zhao *et al.,* 2010; Girlich *et al*., 2007; Huang *et al*., 2009); the same was reported for *Enterococcus* spp. (van den Bogaard *et al*., 2000). A similar situation happens with companion animals: direct interaction between pets and owners enables the contact with the animals' skin, residues of urine and feces, and oral, auricular and nasal secretions. Several reports have documented the presence of fecal multidrug-resistant *E. coli* and *Enterococcus* spp. in dogs and cats (Nam *et al.,* 2010; Wieler *et al*., 2011; Leonard *et al.,* 2012; Hamilton *et al*., 2013) and some of them reported that such multidrug-resistant *E. coli* strains were shared between humans and pets (Stenske *et al.*, 2009; Harada *et al*., 2012) or between cohabitant pets (Leonard *et al.,* 2012) whereas others have postulated that pets could be reservoirs of *Enterococcus* spp. associated with human infections (Damborg *et al*., 2009; Kwon *et al*., 2012; Tremblay *et al*., 2013).

More recently, the environmental pathways were recognized as important routes for AMR spread between different biomes (van Elsas *et al.,* 2011). In addition, antibiotics used in human and veterinary medicine may contaminate the environment via wastewater treatment plant effluents, hospital and processing plant effluents, application of agricultural wastes and biosolids to fields, and leakage from waste-storage containers and landfills (Williams *et al*., 2005; da Costa *et al*., 2008; Kümmerer, 2009; Chagas *et al*., 2011; Wang *et al.,* 2012). Thus, the emergence of resistant pathogens could occur distantly from the original place where such drugs were used and a long time after the original selection pressure. The pool of antibiotic resistant microorganisms in the environment is thought to

be enormous and capable of sharing genes and resistance mechanisms (Martínez, 2012; Perry and Wright, 2013) between them. Every use of an antimicrobial drug has provided the selective pressure necessary to capture, accommodate and turn these complex structures functional, not affecting the bacterial fitness in diverse environments (van Elsas *et al.,* 2011). Presently, this ecological framework is receiving much more attention, with research focused on the assessment of all pathways of indirect transmission, which may be very broad (*e.g.* water cycle) or narrow (*e. g*. hand contact surfaces in hospitals (Kramer *et al*., (2006)). Various studies have reported dissemination of multidrug-resistant microorganisms through veterinary clinical settings (Murphy *et al*., 2010; Hamilton *et al*., 2012; Kukanich *et al*., 2012).

1.1.2. The importance of *Escherichia coli* and enterococci

Few microorganisms are as versatile as *E. coli*. Most strains are harmless and an important part of the normal intestinal microflora of humans and other mammals, being able to compete with the abundant facultative anaerobe intestinal microflora (Kaper *et al*., 2004). However, there are several highly adapted *E. coli* clones that have acquired specific virulence attributes, which confers an increased ability to adapt to new niches and allow them to cause a broad spectrum of disease. These virulence traits, frequently encoded on genetic elements that can be shifted into different strains to create novel combinations of virulence factors, or on genetic elements that might once have been mobile, but have now evolved to become 'locked' into the genome. Only the most successful combinations of virulence factors have persisted to become specific pathotypes of *E. coli* that are capable of causing disease in healthy individuals (Kaper *et al.*, 2004). Extra-intestinal pathogenic *E. coli* (ExPEC), despite being part of the intestinal microflora of a fraction of the healthy population, they can reach and colonize niches outside of the gut, causing disease such as urinary tract infection (UTI), septicemia or meningitis in newborns, as well as UTI or systemic disease in many animals (Köhler and Dobrindt, 2011). Although the host fecal flora is usually the immediate source of ExPEC strains, external reservoirs from which hosts can acquire such strains, as well as the relevant mechanisms of transmission, are still poorly understood (Johnson *et al*., 2008).

Similar to the acquisition of virulence attributes, the evolution of resistance reflects the genomic plasticity of *E. coli*, which results from the frequent acquisition and loss of genomic information as well as the high recombination rates within the flexible genome

(Brzuszkiewicz *et al*., 2009; Tenaillon *et al.,* 2010). Such features make this bacterium an important “indicator” that could be used to track the evolution and dissemination of antibiotic resistance in different ecosystems (van den Bogaard and Stobberingh, 2000; Sáenz *et al*., 2004; Costa *et al.,* 2008a; Murphy *et al.*, 2009).

Enterococci are also commensals of the intestinal microbiota of people and animals; however, they have emerged as one of the most prevalent nosocomial pathogens worldwide, mostly due to their metabolic versatility and intrinsic resistance to inhospitable conditions, which allow them to extensively colonize different environments. Although unable to form spores, enterococci are highly tolerant to desiccation and can persist for months on dried surfaces. Enterococci also tolerate extremes of pH, ionizing radiation, osmotic and oxidative stresses, high heavy metal concentrations, and antibiotics (Ramsey *et al.,* 2014). Moreover, enterococci can also survive or grow over a wide range of temperatures for mesophilic bacteria, from 10 to 45°C. Finally, some strains of enterococci have emerged worldwide as multidrug-resistant and hospital-acquired pathogens (Damborg *et al*., 2009; Ghosh *et al*., 2011; Kwon *et al*., 2012; Tremblay *et al*., 2013; Werner *et al.,* 2013). The species of the highest clinical importance are *Enterococcus faecalis* and *Enterococcus faecium*. Generally, the resistance characteristics of these two species can be categorized as intrinsic resistance, acquired resistance, and tolerance (Kristich *et al.*, 2014).

Although the prevalence of human hospital-acquired enterococci infections is being assessed, little is known about the prevalence of enterococci infections acquired in veterinary hospitals and clinics. Gosh *et al*., (2011) found that dogs discharged from intensive care units on antimicrobial treatment, harboured a large community of multidrug-resistant enterococci. These were probably originated from the endogenous flora of animals with compromised immunity or from the environmental bacteria (KuKanich *et al.*, 2012).

In addition, *E. coli* and *Enterococcus* spp. are able to enter into various transmission cycles, such as: i) the in- and through-household intra- and inter-species transmission; ii) have the ability to exchange resistance genetic determinants with a broad diversity of microbial flora, and iii) survive in the environment (objects, surfaces, food) for enough time to have the opportunity to colonize a new host. In Portugal, multidrug-resistant *E. coli* were recently identified in feces of seagulls (Poeta *et al*., 2008; Simões *et al*., 2010), wild boars (Poeta *et al*., 2009) and other wild animals (Costa *et al*., 2008b). Moreover, multidrug-resistant isolates of *Enterococcus* spp. and *E. coli* were recovered from 30 fecal samples of the wild Iberian lynx from South Spain (Gonçalves *et al*., 2013).

There are currently irrefutable evidences of distant AMR dissemination, such as the findings of antimicrobial-resistant strains in animals from inhospitable places worldwide, as in the Arctic birds (Sjölund *et al*., 2008) or in the Iguanas from Galapagos Islands (Thaller *et al*., 2010).

Taking into account the previous considerations, *E. coli* and *Enterococcus* spp. are invaluable bacteria to assess the burden of antibiotic resistance within a certain population. Regular monitoring of the level of AMR in pathogens and normal flora has been recommended by the World Health Organization since 2001 at WHO Global Strategy for Containment of AMR (WHO 2012b). Some national and international surveillance programs on AMR have been established for people as well as for food-producing animals (SENTRY, SCOPE, SWEDRES, SVARM, FAO, DANMAP and NARMS) although pet animals have been ordinarily excluded from such programs (Gosh *et al*., 2011).

1.1.3. The role of companion animals

A crucial importance has been attributed to the transmission of multidrug-resistant bacteria (or genetic material) between food-producing animals and humans, while little attention has been given to the contribution of companion animals to the scenario of AMR. It is expectable that dogs and cats, by sharing the same household, being exposed to the same substances and contacting with the same objects and surfaces as their owners, influence the AMR status of the domestic aggregate. Therefore, such pets play a role in the supply of bacteria to the household pool of microorganisms, hence contributing to the spread and even share of household antimicrobial resistant bacteria or genetic determinants.

During the last decades, a change in the social role of companion animals has taken place, resulting in closer contacts between owners and their pets. The evolution of veterinary practice, the improved living conditions of the community and the increased sense of social responsibility for the welfare and health of pets have conducted to an extended pets' longevity with a substantial augment of oncologic and geriatric patients, more prone to chronic, debilitating and immunocompromising diseases and in need for antimicrobial treatments (da Costa *et al.*, 2013).

Portugal accompanied the global veterinary medicine evolution and has, at the present time, 4798 active veterinary practitioners and 1240 approved small animal veterinary attendance centers (CAMVs) (OMV, 2014). In the recent years, more and new veterinary approved antimicrobial formulations became available to the Portuguese professionals. Nowadays, Portugal has 168 approved antimicrobial medicament presentations for veterinary use (DGAV, 2014) with quinolones comprising almost half of them (47.0%), whereas amoxicillin and clavulanic acid constitute 24.4% and cephalosporins 11.3% (all first generation with one exception: cefovecin). According to DGAV data (2010), during the last years massive quantities of veterinary antimicrobials were consumed in Portugal, reaching a maximum in 2010 (179,874 kilograms) with a gradual decline since then (158,906 kilograms in 2012). Unfortunately, detailed data are unavailable, hampering the in depth analysis of the specific use of antimicrobials in the Portuguese veterinary field. Examples are the lack of information about which antimicrobials are the most administered to a particular species or to what extent are antibiotics used in human medicine also administered to companion animals.

The general focus on agriculture and food-producing animals as a source of resistant bacteria and resistance genes for human pathogens may underestimate the role of companion animals as one of the contributors to resistance in human pathogens. However, the close contact between companion animals and humans builds up a unique and critical aspect related to antimicrobial resistance that creates opportunities for inter-species transmission of multidrug-resistant bacteria (EMA, 2013). Furthermore, similar to human medicine, the high prevalence of pets' infections by resistant microorganisms is limiting the veterinary therapeutic options. In fact, resistant strains to last-line antibiotics of exclusive human use, such as carbapenems, were already recovered from companion animals (Shaheen *et al*., 2013). Therefore, veterinarians play an important role in the global approach for combating AMR. The assessment of the real need for antimicrobial treatment; rational and appropriate choice of the drugs; knowledge of the resistance transmission pathways; sharing of surveillance data; and animal owners information on preventive measures during and after antimicrobial treatment are important stress points in the clinical activity of small animal practitioners that are essential in such context.

In summary, antimicrobial resistance is a kind of snow ball that is rolling down the hill and embodying everyone that is nearby.

1.2. RATIONALE AND AIMS

One of the current challenges in AMR is to assess the public health burden that companion animals' resistant bacteria or resistance genes represent. This assessment is made difficult by the lack of data, as well as by the fact that transmission of antimicrobial resistance is a complex and largely unpredictable phenomenon involving different routes and mechanisms.

Given the importance of antibiotics for human and animal health, this difficulty cannot be considered an insurmountable obstacle. Rather, it should be understood as an object of study for which every single contribution is important.

Two purposes were planned for the present work:

I – A survey study of the antimicrobial resistance profiles of fecal *E. coli* and *Enterococcus* spp. from domestic dogs and cats in Portugal and the estimation of risk factors for antimicrobial resistance development;

II – The assessment of the spread and share of antimicrobial resistant determinants or bacteria within household, comprising human and pet cohabitants and common touched objects and surfaces.

Chapter 2

ANTIMICROBIAL RESISTANCE PREVALENCE AND RISK FACTORS

HOUSEHOLD ANTIMICROBIAL RESISTANCE SHARE AND SPREAD

2.1. ANTIMICROBIAL RESISTANCE PREVALENCE AND RISK FACTORS

2.1.1. Paper I

PREVALENCE OF ANTIMICROBIAL RESISTANCE IN ENTERIC *ESCHERICHIA COLI* FROM DOMESTIC PETS AND ASSESSMENT OF ASSOCIATED RISK MARKERS USING A GENERALIZED LINEAR MIXED MODEL

Leite-Martins, L., Mahú, M.I., Costa, A.L., Mendes, A., Lopes, E., Mendonça, D.M.V., Niza-Ribeiro, J., de Matos, A.J.F., da Costa, P.M

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Prevalence of antimicrobial resistance in enteric *Escherichia coli* from domestic pets and assessment of associated risk markers using a generalized linear mixed model

Liliana Leite-Martins [a,*], Maria I. Mahú [b], Ana L. Costa [b], Ângelo Mendes [b], Elisabete Lopes [b], Denisa M. V. Mendonça [c, d], João Niza-Ribeiro [c, d], Augusto J. F. de Matos [a], Paulo Martins da Costa [b]

[a] Veterinary Clinics Department, Abel Salazar Institute for the Biomedical Sciences (ICBAS), Porto University (UP), Portugal

[b] Microbiology and Food Technology Department, Abel Salazar Institute for the Biomedical Sciences (ICBAS), Porto University (UP), Portugal

[c] Population Studies Department, Abel Salazar Institute for the Biomedical Sciences (ICBAS), Porto University (UP), Portugal

[d] Public health Institute (ISPUP), Porto University (UP), Portugal

Abstract

Antimicrobial resistance (AMR) is a growing global public health problem for which the use of antimicrobials both in human and animal medical practice have an important contribution. The objectives of the present cross-sectional study were: 1) to determine the prevalence of resistance in *Escherichia coli* isolated from feces of pets from Porto region, in Portugal, against 19 antimicrobial agents and 2) to assess individual, clinical and environmental characteristics associated with each pet as risk markers for the AMR found in *E. coli* isolates.

From September 2009 to May 2012, rectal swabs were collected from pets selected using a systematic random procedure from the ordinary population of animals attending the Veterinary Hospital of Porto University. A total of 78 dogs and 22 cats were sampled with the aim of isolating *E. coli*. Animal owners, who allowed the collection of fecal samples from their pets, answered a questionnaire to collect information about the markers that could influence the AMR of enteric *E. coli*. Chromocult tryptone bile X-glucuronide agar was employed for *E. coli* isolation and disc diffusion method was used to determine antimicrobial susceptibility. The data were analyzed using a multilevel, univariable and multivariable generalized linear mixed model (GLMM). Several (49.7%) out of the 396 isolates obtained in this study were multidrug-resistant. Antimicrobial agents for which many *E. coli* isolates exhibited resistance were ampicillin (51.3%), cephalothin (46.7%), tetracycline (45.2%) and streptomycin (43.4%). Previous quinolone treatment was the main risk marker for the presence of AMR in 12 (ampicillin, cephalothin, ceftazidime, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, tetracycline, streptomycin, chloramphenicol, trimethoprim-sulfamethoxazol and aztreonam) out of the 15 antimicrobials assessed. Coprophagic habits were also positively associated with an increased risk of AMR for 6 drugs: ampicillin, amoxicillin-clavulanic acid, cephamycin, ciprofloxacin, streptomycin, and trimethoprim-sulfamethoxazol.

In summary, pets with record of one or more previous treatments with quinolones and exhibiting coprophagic habits were at increased risk of harboring multidrug-resistant *E. coli* strains in their feces when compared with pets having not such characteristics. AMR is a serious global problem and assessing the risk markers for the presence of drug-resistant bacteria in pets, a very close source of resistance determinants to humans, is essential for the implementation of safe handling procedures for companion animals and prudent selection of antimicrobial substances in veterinary practice.

Keywords: Antimicrobial resistance; pets; *Escherichia coli*; prevalence; risk markers.

1. Introduction

Antimicrobial resistance (AMR) will probably be one of the main global public health problems of the next decade (Carlet et al., 2012). The phenomenon of AMR, which is based on the genetic plasticity of bacteria, has the selective pressure exerted by the antimicrobial usage in human and veterinary medicine, animal production, fish production, agriculture and food technology, the main driver force for its emergence (Kearns, 2010; EAAD, 2013; Martins da Costa et al., 2013). Resistant bacteria may be transmitted between interdependent hosts and spread into the environment, contributing to the worldwide increase of AMR (CDC, 2013). The progress in veterinary medicine and the number of domestic pets treated by specialized practitioners generated an increased usage of antimicrobial treatments (Martins da Costa et al., 2013). Additionally, pets live longer and are in closer contact with their owners, favoring the mutual transfer of microbial flora, directly by skin or bacteria-containing material contact (e.g. saliva and feces) and indirectly, via the household environment (Martins et al., 2013). When reaching the new host, resistant bacteria can colonize, infect, or remain in that particular environment for very short periods of time. In all cases, resistant bacteria can either spread their resistance genes to host-resident bacteria (commensals or pathogens) or accept resistance genes from such microorganisms (Jernberg et al., 2010). As a consequence, AMR in companion animals is simultaneously an important veterinary medical issue and a public health concern (Lloyd, 2007).

The regular monitoring of AMR in pathogenic and normal flora has been recommended by the World Health Organization and the European Centre for Disease Prevention and Control. For this purpose, the European Antimicrobial Resistance Surveillance Network (EARS-Net), involving 53 countries, was created (EFSA and ECDPC, 2013). Similar programs have been proposed for veterinary medicine, leading to the development of field studies on food animals (Aarestrup, 2004; Taylor et al., 2008) and pets (Moyaert et al., 2006; Lloyd, 2007; Costa et al., 2008; Murphy et al., 2009; Leonard et al., 2012). However, to our knowledge, no studies have included clinical histories of both pets and their cohabitants neither household features in order to assess potential AMR risk markers.

Escherichia coli is an important member of the normal intestinal microflora of humans and other mammals, but it can be also a highly versatile pathogen, causing diverse intestinal and extra intestinal diseases by means of virulence factors that affect a wide range of cellular processes (Kaper et al., 2004). Carriage of AMR has been

associated with several treatment failures in both human and veterinary patients (Toutain et al., 2010; Vigil et al., 2009).

The present study intended to determine the proportion of antimicrobial resistance of *E. coli* isolated from feces of pets from Porto region, in Portugal, as well as to assess individual, clinical and environmental characteristics of pets as risk markers for the AMR found in the isolated strains. It is hypothesized that animals with relevant clinical background will harbor more resistance *E. coli* isolates.

2. Materials and methods

2.1. Enrollment and sampling

A random systematic approach was used to select animals to the present cross-sectional study, performed at the Veterinary Hospital of Porto University (UPVET). From September 2009 to May 2012, on Monday or Tuesday, one among the first five pets to arrive at the UPVET attending room was randomly selected to be included in the study. If the owners refused to collaborate in the study the next pet, by order of arrival, was included, without following any criteria. To be eligible to be enrolled in the study, the animal should not have received any antimicrobial therapy within the preceding 4 months. The owners were asked to sign a consent form, to fill a questionnaire and to allow the collection of fecal samples from their pets using rectal swabs. Approval to conduct the study was previously obtained from the Ethics Committee of the Abel Salazar Institute for the Biomedical Sciences, University of Porto.

2.2. Questionnaire

By a brief questionnaire, owners were asked to provide information about possible risk markers for multidrug-resistant (Magiorakos et al., 2012) *E. coli* colonization. The questionnaire was constructed following similar studies in animals (Akwar et al., 2007; Ahmed et al., 2012; Boothe, 2012) and humans (McDonald et al., 2001; Sotto et al., 2001; Lietzau et al., 2007; Kalter et al., 2010; Lastours et al., 2010). To evaluate the potential risk markers, questionnaires included individual and clinical characteristics, such as 1) species, 2) gender, 3) age, 4) daily access to the outside environment (indoor habitat refers to those animals which live predominantly at home or with very restricted access

outdoor), 5) diet ("commercial" refers to the animals that were fed with strictly commercial dry or wet food), 6) coprophagic habits (ingestion of feces, both their own or from other animals), 7) previous systemic antimicrobial treatments with particular emphasis on 8) previous systemic quinolone treatments (assessed through the clinical file of the pet), 9) existence of cohabitant pets in the household, 10) previous antimicrobial treatments received by owners, 11) owners' professional connection with healthcare units such as human or veterinary hospitals, clinics or health centers (such elements were classified as "Health Professionals", 12) "reason for veterinary visit" (recorded by the veterinary surgeon following a complete physical examination).

2.3. *Escherichia coli* isolation

Fecal samples were obtained using saline wet swabs that were introduced with circular movements into the rectum of each animal. Swabs were immediately immersed on Buffered Peptone Water (BPW) (Oxoid, Basingstoke, Hampshire, England), transported to the laboratory and stored at room temperature for 1 h. Then, for *E. coli* isolation, an aliquot of 5 µl was streaked on Chromocult tryptone bile X-glucuronide (TBX) agar (Biokar Diagnostics, Allonne, Beauvais, France) and incubated at 37 °C for 24 h. Two to five confirmed pure colonies with typical appearance of *E. coli* were selected on the basis of colony size and morphology. The described procedure and the biochemical confirmation of isolates were adapted from standard protocols used in similar studies, aiming to achieve the most reliable and accurate *E. coli* detection (Costa et al., 2008; Simões et al., 2010; Martins et al., 2013).

2.4. Antimicrobial susceptibility characterization

Disk diffusion assay, following standard guidelines (CLSI, 2012), was performed to assess the antimicrobial susceptibility of each isolate. Antimicrobial drugs were selected in order to include those regularly used in both human and veterinary medicine and to provide diversity by representing different antimicrobial classes (Goossens et al., 2005; Elseviers et al., 2007; EFSA and ECDPC, 2013). A total of 19 antimicrobial agents (AM) (Oxoid, Basingstoke, Hampshire, England) were used: ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg), cephalothin (CEF, 30 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), nalidixic acid (NAL, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), tetracycline (TET, 30 µg), streptomycin (STR, 10 µg), amikacin (AMK, 30 µg), trimethoprim-sulfamethoxazol (SXT,

25 µg), chloramphenicol (CHL, 30 µg), tobramycin (TOB, 10 µg), kanamycin (KAN, 30 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), and nitrofurantoin (NIT, 300 µg). The interpretation of the inhibition zone length followed the Clinical and Laboratory Standards Institute (CLSI) recommendations and breakpoints for *Enterobacteriaceae* (CLSI, 2012).

2.5. Data analysis

The prevalence of AMR regarding each AM was calculated by dividing the number of resistant *E. coli* isolates by the total number of *E. coli* tested. The potential risk markers obtained from the questionnaire were analyzed as categorical variables as follows: dichotomous variables, such as species (canine, feline), gender (male, female), reason for veterinary visit (routine check-up, illness signs), habitat type (indoor, mixed), diet (commercial, mixed), previous quinolone treatments (yes, no), health professionals owners (yes, no), owners submitted to previous antimicrobial treatments (yes, no), cohabitant pets (yes, no), coprophagy habits (yes, no); the exposure of the animal to any previous antimicrobial treatment was transformed into a categorical variable with three levels: “none”, “just one”, and “two or more”. Age was also categorized with three levels: “young” (before 2 years of age), “adult” (between 2 and 10 years old), and “old” (with more than 10 years old). The outcome in the analysis was the result of AMR which was dichotomized in either resistant or sensitive; intermediate results were categorized as sensitive. Using the European Food Safety Authority criteria, each antimicrobial was further classified into one of the following categories of prevalence of AMR: extremely high: >70%; very high: 50-70%; high: 20-50%; moderate: 10-20%; low: 1-10%; very low: 0.1-1% and rare: <0.1% (EFSA and ECDPC, 2013).

A descriptive analysis of AMR prevalence and frequency of risk markers was conducted. To analyze these markers and to assess the strength of their association with the AMR, a Multilevel Generalized Linear Mixed Model (GLMM) was used.

The logit link function was used to model the probability of occurrence of resistance to an antibiotic. To take into account the multilevel structure of the data in which more than one *E. coli* strain (*i*) was isolated from each animal (*j*), a two level structure in the data was assumed in which *E. coli* strains (first level) were nested within the animal from which they were isolated (second level).

The data were modeled in the following way:

$Y = \begin{cases} 0\ (no\ AMR) \\ 1\ (AMR) \end{cases}$ Where Y is the response variable.

Pr(Y) = pij, i = 1, …, 396 and j = 1, ..., 100.

The generic model used the following equation: $logit(p_{ij}) = a + c_j + \beta\ \ animal\ variables_j$ The model, the animal (the pet) was allowed to be random. The second level random effect is given by $c_j \sim N(0,\sigma 2)$ where σ2 is the variance of the random effects at the animal level.

The basic multivariable multilevel model was as follows:

$$\begin{aligned} logit(p_{ij}) = a + c_j + \beta_1 Species_j + \beta_2 Age_j + \beta_3 Gender_j + \beta_4 Reason\ of\ visit_j + \beta_5 Habitat_j \\ + \beta_6 Diet_j + \beta_7 Number\ AM\ treatments_j \\ + \beta_8 Previous\ Quinolones\ treatments_j + \beta_9 Owner's\ profession_j \\ + \beta_{10} Owner's\ AM\ treatments_j + \beta_{11} Cohabitants\ Pets_j \\ + \beta_{12} Coprophagy\ habits_j \end{aligned}$$

Variables codes are presented in Tables 4 to 6.

A three step procedure was taken as follows: firstly, a univariable multilevel GLMM analysis was conducted to assess the individual relationship between each potential risk factor and the presence of AMR; a second step was performed to conduct a multivariable multilevel GLMM analysis with all the variables that had a $p < 0.15$ in the previous analyses followed by a manual backward and forward procedure to obtain a final model where each factor effect was adjusted for the remaining factors. Only factors with a $p < 0.05$ were retained in the final model. The data were analyzed using the procedure GEE in the SPSS Software V. 21.0 (IBM SPSS statistical 21 package, IBM Corporation, NY).

3. Results

A total of 78 dogs and 22 cats belonging to 100 distinct households were enrolled. Overall 396 *E. coli* isolates were obtained, 307 (77.5%) were isolated from dogs and 89 (22.5%) from cats, ranging from two to five per animal; on average, 3.96 isolates per animal were obtained.

3.1. Antimicrobial resistance profiles

Our results showed that 28.8% of the isolates were susceptible to all compounds; the median number of AMR among the isolates was three and the isolate in the 75th percentile harbored seven resistances. Extreme resistance towards 14 or 15 AM was present in five isolates (1,3 %). The histogram displaying the absolute number of resistances suggests the existence of two or perhaps three subpopulations of *E. coli* (Fig. 1): one group with less than four resistances, a second one with five to 10 resistances, and a conceivable third group with more than 10 resistance results.

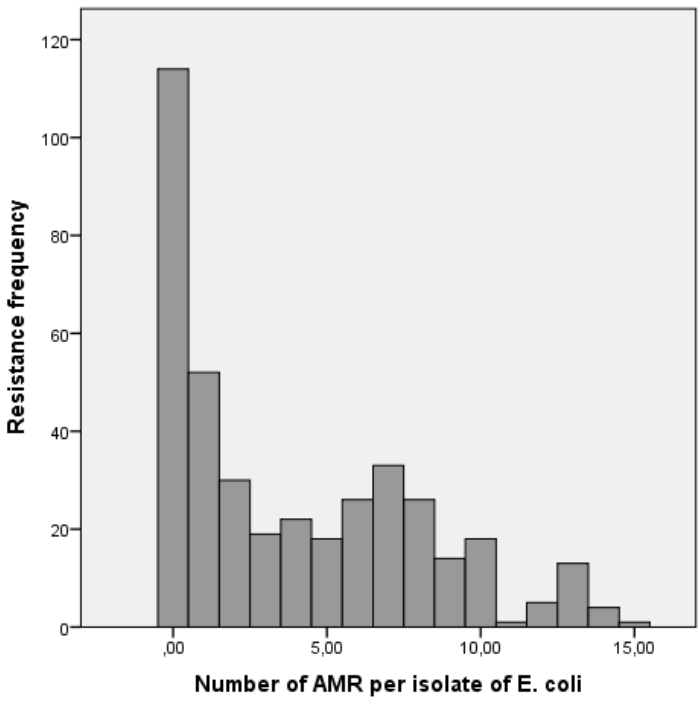

Fig.1. Frequency of antimicrobial resistance in *Escherichia coli* isolates (n=396).

3.2. Antimicrobial resistance prevalence

The prevalence of AMR varied from 0% found for nitrofurantoin and imipenem up to 51.3% (+/- 0.049) for ampicillin. After categorization according to the EFSA (EFSA and ECDPC, 2013) recommendations, 5.3% (+/- 0.022) of the tested AM were in the very high resistance category; 31.6% (+/- 0.046) in the high resistance group and a similar proportion were in the moderate resistance category, as displayed in Table 1.

3.3. Distribution of potential risk markers associated with pets

The frequency of each tested potential risk marker is shown in Table 2. After comparing the factors species, age, sex and reason for the visit, it was clear that the population of pets enrolled in our study resembles the population of cats and dogs attending the hospital. Twenty-three dogs (29.5 %) and 15 cats (68.2%) were healthy animals admitted for regular check-up or prophylactic actions, while the remaining animals attended the hospital for clinical reasons.

3.4. Distribution of potential risk markers among *E. coli* isolates

The distribution of potential risk markers amongst *E. coli* isolates are displayed in Table 3. The largest numbers of isolates were obtained from pets owned by non-health professionals (n = 304; 76.8%) and animals with outdoor access (n = 302; 76.3%). Some characteristics associated with categories with small proportion of isolates were having just one antimicrobial treatment (n = 81; 20.5%), being older than 10 years (n = 93; 23.5%) and living indoor (n =9 4; 23.7%).

Table 1. Categorization of the tested antimicrobials (AM) in *Escherichia coli* isolates according to EFSA risk categories for prevalence of resistances.

EFSA Risk Categories	AM	Prevalence of resistance	+/- C.I.	Frequency of AM in each category	+/- C.I.
Extremely High		0.0	-	0.0	-
Very High	AMP	51.3	0.049	5.3	0.022
High	CEF	46.7	0.049	31.6	0.046
	NAL	35.9	0.047		
	CIP	29.5	0.045		
	TET	45.2	0.049		
	STR	43.4	0.049		
	SXT	36.4	0.047		
Moderate	AMC	12.1	0.032	31.6	0.046
	CAZ	13.6	0.034		
	CTX	14.6	0.035		
	CHL	18.2	0.038		
	KAN	13.9	0.034		
	ATM	17.7	0.038		
Low	FOX	5.8	0.023	15.8	0.036
	GEN	5.8	0.023		
	TOB	3.0	0.017		
Very Low	AMK	0.5	0.017	5.3	0.022
Rare	NIT	0.0	-	10.5	0.030
	IPM	0.0	-		

Legend: AM – antimicrobial agent; C.I. – Confidence interval; AMP – ampicillin; AMC – amoxicillin-clavulanic acid; CEF – cephalothin; FOX – cephoxitin; CAZ – ceftazidime; CTX – cefotaxime; NAL – nalidixic acid; CIP – ciprofloxacin; GEN – gentamicin; NIT – nitrofurantoin; TET – tetracycline; STR – streptomycin; AMK – amikacin; SXT – trimethoprim-sulfamethoxazol; CHL – chloramphenicol; TOB – tobramycin; KAN – kanamycin; IPM – imipenem; ATM – aztreonam. Values are expressed in percentages.

Table 2. Distribution of potential risk marker categories among pets (n=100).

Risk marker	Category	Dogs	% Dogs	Cats	% Cats	Dogs + Cats
Species		78		22		100
Age	< 2 years	24	30.8	9	40.9	33
	2 - 10 years	34	43.6	11	50.0	45
	> 10 years	20	25.6	2	9.1	22
Gender	Female	47	60.3	9	40.9	56
	Male	31	39.7	13	59.1	44
Reason for veterinary visit	Check up	23	29.5	15	68.2	38
	Illness	55	70.5	7	31.8	62
Habitat Type	Indoor	9	11.5	15	68.2	24
	Mixed	69	88.5	7	31.8	76
Diet	Commercial	25	32.1	10	45.5	35
	Mixed	53	67.9	12	54.5	65
Animal Antimicrobial Treatments	None	21	26.9	17	77.3	38
	Just One	19	24.4	3	13.6	22
	Two/more	38	48.7	2	9.1	40
Animal Quinolone Treatments	Yes	28	35.9	2	9.1	30
	No	50	64.1	20	90.9	70
Owners Health Professionals	Yes	16	20.5	6	27.3	22
	No	62	79.5	16	72.7	78
Owners Antimicrobial Treatments	Yes	38	48.7	6	27.3	44
	No	40	51.3	16	72.7	56
Cohabitant Pets	Yes	36	46.2	16	72.7	52
	No	42	53.8	6	27.3	48
Coprophagy Habits	Yes	29	37.2	4	18.2	33
	No	49	62.8	18	81.8	67

Table 3. Distribution of the *Escherichia coli* isolates (n=396) of Canine (n=307) and Feline (n=89) origin by potential risk marker categories.

Risk Marker	Category	Canine Isolates	Feline Isolates	Total Isolates (%)
Age	< 2 years	85	32	117 (29.5)
	2 - 10 years	135	51	186 (47.0)
	> 10 years	87	6	93 (23.5)
Gender	Female	185	37	222 (56.1)
	Male	122	52	174 (43.9)
Reason for veterinary visit	Check up	81	64	145 (36.6)
	Illness	226	25	251 (63.4)
Habitat Type	Indoor	35	59	94 (23.7)
	Mixed	272	30	302 (76.3)
Diet	Commercial	107	34	141 (35.6)
	Mixed	200	55	255 (64.4)
Animal Antimicrobial Treatments	None	71	67	138 (34.8)
	Just One	69	12	81 (20.5)
	Two or more	167	10	177 (44.7)
Animal Quinolone Treatments	Yes	121	10	131 (33.1)
	No	186	79	265 (66.9)
Owners Health Professionals	Yes	65	27	92 (23.2)
	No	242	62	304 (76.8)
Owners Antimicrobial Treatments	Yes	162	25	187 (47.2)
	No	145	64	209 (52.8)
Cohabitant Pets	Yes	142	66	208 (52.5)
	No	165	23	188 (47.5)
Coprophagy Habits	Yes	119	18	137 (34.6)
	No	188	71	259 (65.4)

3.5. Antimicrobial resistance and potential risk markers

The frequencies of AMR for each potential risk marker are displayed in Table 4. None isolate displayed resistance to nitrofurantoin or imipenem; therefore these two AM were excluded from further analyses. The AMR proportions were calculated based on all isolates (n = 396).

Table 4. Proportion of antimicrobial resistance (%) distributed by potential risk marker categories.

Risk Markers	**AMP**	**AMC**	**CEF**	**FOX**	**CAZ**	**CTX**	**NAL**	**CIP**	**GEN**	**TET**	**STR**	**SXT**	**CHL**	**KAN**	**ATM**
Species Canine	58.3	15.0	53.1	7.5	14.7	18.9	39.1	35.2	7.2	47.6	45.6	39.4	20.2	14.0	20.8
Species Feline	27,0	2.2	24.7	0.0	10.1	0.0	24.7	10.1	1.1	37.1	36.0	25.8	11.2	13.5	6.7
Age: < 2 years	47,0	2.6	37.6	0.0	11.1	14.5	25.6	25.6	1.7	35.9	35.0	30.8	12.8	6.0	16.2
Age: 2 - 10 years	44.6	9.1	40.3	5.4	10.2	12.4	35.5	28.5	5.4	45.2	44.6	34.9	18.8	17.2	14.0
Age: > 10 years	69.9	30.1	71.0	14,0	23.7	19.4	49.5	36.6	11.8	57.0	51.6	46.2	23.7	17.2	26.9
Gender Female	45.0	10.8	44.1	4.1	11.3	14.4	34.2	27.5	6.3	45.5	40.1	36,0	11.3	14.9	16.7
Gender Male	59.2	13.8	50.0	8.0	16.7	14.9	37.9	32.2	5.2	44.8	47.7	36.8	27.0	12.6	19.0
Reason: Check up	38.6	4.1	30.3	1.4	4.8	3.4	23.4	17.2	1.4	34.5	30.3	33.1	9.7	11.7	6.9
Reason: Illness	58.6	16.7	56.2	8.4	18.7	21.1	43.0	36.7	8.4	51.4	51.0	38.2	23.1	15.1	23.9
Habitat: Indoor	37.2	2.1	33.0	0.0	13.8	4.3	39.4	25.5	2.1	48.9	41.5	23.4	20.2	17.0	11.7
Habitat: Mixed	55.6	15.2	51.0	7.6	13.6	17.9	34.8	30.8	7.0	44.0	44.0	40.4	17.5	12.9	19.5
Diet: Commercial	44.7	12.8	46.1	9.2	16.3	11.3	34.0	24.1	0.0	44.7	34.0	34.0	13.5	5.7	16.3
Diet: Mixed	54.9	11.8	47.1	3.9	12.2	16.5	36.9	32.5	9.0	45.5	48.6	37.6	20.8	18.4	18.4
AM Tx: None	40.6	5.1	32.6	0.7	4.3	6.5	18.1	13.8	3.6	31.2	29.0	29.7	10.9	11.6	6.5
AM Tx: One	45.7	0.0	40.7	0.0	11.1	17.3	44.4	44.4	7.4	48.1	43.2	38.3	19.8	16.0	22.2
AM Tx: 2 or +	62.1	23.2	60.5	12.4	22.0	19.8	45.8	35.0	6.8	54.8	54.8	40.7	23.2	14.7	24.3
Quinolone Tx: Yes	77.1	19.1	69.5	12.2	32.8	33.6	67.2	56.5	13.0	63.4	64.9	51.1	34.4	21.4	42.0
Quinolone Tx: No	38.5	8.7	35.5	2.6	4.2	5.3	20.4	16.2	2.3	36.2	32.8	29.1	10.2	10.2	5.7
O. Prof.: Health Prof.	65.2	23.9	55.4	6.5	7.6	7.6	37.0	34.8	1.1	56.5	55.4	53.3	19.6	10.9	10.9
O. Prof.: Others	47.0	8.6	44.1	5.6	15.5	16.8	35.5	28.0	7.2	41.8	39.8	31.2	17.8	14.8	19.7
O. AM Tx: Yes	54.5	15.0	50.8	6.4	14.4	18.7	43.9	36.9	9.1	47.6	50.8	40.1	19.8	16.0	19.3
O. AM Tx: No	48.3	9.6	43.1	5.3	12.9	11.0	28.7	23.0	2.9	43.1	36.8	33.0	16.7	12.0	16.3
Cohabit. Pets: Yes	51.4	15.9	45.7	7.7	13.0	17.8	38.5	34.1	7.7	46.6	46.6	43.3	20.2	15.4	21.2
Cohabit. Pets: No	51.1	8.0	47.9	3.7	14.4	11.2	33.0	24.5	3.7	43.6	39.9	28.7	16.0	12.2	13.8
Coprophagy: Yes	67.9	26.3	56.9	13.1	19.7	22.6	48.9	42.3	10.9	57.7	57.7	54.0	23.4	16.8	27.7
Coprophagy: No	42.5	4.6	41.3	1.9	10.4	10.4	29.0	22.8	3.1	38.6	35.9	27.0	15.4	12.4	12.4

Legend: AM - antimicrobial; TX - treatment; O. - owner; Prof. - professional; Cohabit.- cohabitant; AMP – ampicillin; AMC – amoxicillin-clavulanic acid; CEF – cephalothin; FOX – cephoxitin; CAZ – ceftazidime; CTX – cefotaxime; NAL – nalidixic acid; CIP – ciprofloxacin; GEN – gentamicin; NIT – nitrofurantoin; TET – tetracycline; STR – streptomycin; SXT – trimethoprim-sulfamethoxazol; CHL – chloramphenicol; KAN – kanamycin; IPM – imipenem; ATM – aztreonam.

The lowest AMR rates were found in young indoor animals nourished with commercial diet and submitted to a single previous antimicrobial treatment. In this latest group, there were no isolates resistant to amoxicillin-clavulanic acid and cephoxitin; no cephoxitin-resistant isolates were found in cats or in young or indoor animals; finally, no cefotaxime-resistant isolates were found in cats and no gentamicin-resistant isolates were recovered from animals fed with commercial diet.

3.6. Results of the multilevel univariable analysis

Table 5 displays the results of the multilevel univariable analysis. Only Odds Ratio (OR) for the variables and categories in which the p value was lower than 0.15 are shown. The markers that presented a significant ($p < 0.05$) increased risk of resistance were the following: being a dog (for ampicillin, cephalothin and ciprofloxacin); previous exposure to quinolone treatments (for ampicillin, cephalothin, ceftazidime, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, tetracycline, streptomycin, trimethoprim-sulfamethoxazol, chloramphenicol and aztreonam); pets being owned by health professional workers (for amoxicillin-clavulanic acid); or having coprophagic habits (for ampicillin, amoxicillin-clavulanic acid, cephoxitin, nalidixic acid, ciprofloxacin, tetracycline, streptomycin, trimethoprim-sulfamethoxazol and aztreonam). Protective markers were the following: young age(with less than 10 years old), for ampicillin, amoxicillin-clavulanic acid and cephalothin; being a female for chloramphenicol; commercial diet, for kanamycin; being presented for checkup, for ampicillin, amoxicillin-clavulanic acid, cephalothin, cefotaxime, nalidixic acid, ciprofloxacin, tetracycline, streptomycin and aztreonam; and, absence of previous antimicrobial treatments, for ampicillin, amoxicillin-clavulanic acid, cephalothin, ceftazidime, nalidixic acid, ciprofloxacin, tetracycline, streptomycin and aztreonam. Only in the case of amikacin and tobramycin there was not found any significant risk marker association with AMR and for this reason they were not included in the subsequent multilevel multivariable analysis.

3.7. Results of the multilevel multivariable analysis

The final model, obtained with a multilevel multivariate analysis after manual backward and forward variable selection, resulted in a robust model retaining only the variables that, after adjustment for all the other variables remained significant at $p < 0.05$ (Table 6). In this analysis, ampicillin was the antimicrobial agent whose resistance was

significantly associated with the highest number of markers. 5 out of 12 (“species”, “gender”, “previous quinolone treatment”, “health professionals owners” and “coprophagic habits”). Resistances to amoxicillin-clavulanic acid and chloramphenicol were both significantly associated with three and two different markers, respectively. Resistance to ciprofloxacin, streptomycin and trimethoprim-sulfamethoxazol showed an association with two similar markers (“previous quinolone treatment” and “coprophagic habits”), whereas AMR to cephalothin, ceftazidime, cefotaxime, nalidixic acid, gentamycin, tetracycline, and aztreonam retained one significant association (“previous quinolone treatment”). Finally, cephoxitin resistance was associated with coprophagy and kanamycin with mixed diet.

“Previous quinolone treatments” and “coprophagic habits” were significantly related with AMR in 12 and 6 out of the 15 antimicrobial agents tested, respectively. According to the model, pets that had been submitted to prior quinolone treatments have a significant high risk of being colonized by *E. coli* resistant to ceftazidime, OR 16.78, (2.33-120.74); cefotaxime, OR 22.01, (3.15-154.01); nalidixic acid, OR 13.51, (3.83-47.61) and aztreonam, OR 19.18, (3.67-100.14). Animals with coprophagic habits are at a higher risk of harboring *E. coli* isolates resistant to amoxicillin-clavulanic acid OR 10.35, (2.68-40.59) and cephoxitin, OR 11.21, (1.26-99.64). (Table 6). The significance level of each OR can be read from Table 6, by the number of asterisks associated: * - < 0.05; ** - < 0.01; *** - < 0.001.

Overall, the risk markers significantly associated with AMR were: i) previous treatment with quinolones (12 out of 15) and, ii) coprophagic habits (6 out of 15). The other variables were only sporadically associated with some AMR: i) canine species (ampicillin); ii) male gender (ampicillin, chloramphenicol); iii) illness (amoxicillin-clavulanic acid and chloramphenicol); iv) mixed diet (kanamycin) and v) health professionals owners (ampicillin, amoxicillin-clavulanic acid).

Table 5. Risk markers for antimicrobial resistance of *E. coli* isolates from the univariable multilevel analysis.

Risk Markers	AMP	AMC	CEF	FOX	CAZ	CTX	NAL	CIP	GEN	TET	STR	SXT	CHL	KAN	ATM
	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR
Species Canine	6.63**	7.08	5.37**				2.43	5.83*							4.76
Species Feline															
Age: < 2 years	0.3	0.06**	0.14**		0.42		0.26	0.53		0.29					
Age: 2 - 10 years	0.24*	0.17*	0.16**		0.28		0.46	0.61		0.52					
Age: > 10 years															
Gender Female	0.4												0.25*		
Gender Male															
Reason: Check up	0.30*	0.22*	0.23**		0.26	0.14*	0.35*	0.32*		0.36*	0.30*		0.36		0.23*
Reason: Ilness															
Habitat: Indoor	0.31	0.13	0.33			0.16						0.38			
Habitat: Mixed															
Diet: Commercial											0.42			0.24*	
Diet: Mixed															
AM Tx: None	0.30*	0.19*	0.21**	0.10	0.17*	0.26	0.20**	0.25*		0.24*	0.22**				0.19*
AM Tx: One	0.35	0,00	0.31	0.00	0.41	0.78	0.89	1.59		0.66	0.475				0.97
AM Tx: 2 or +															
Quinolone Tx: Yes	10.01***		7.71***	4.5	15.94***	13.84***	14.92***	12.28***	4.73*	4.76**	6.98***	3.43*	6.42**	2.4	20.79***
Quinolone Tx: No															
O.Prof.:Health Prof.	2.7	4.69*													
O. Prof.:Others															
O. AM Tx: Yes							2.51	2.23			2.65				
O. AM Tx: No															
Cohabit. Pets: Yes															
Cohabit. Pets: No															
Coprophagy: Yes	4.14**	10.43**	2.39	10.98*		3.22	3.22*	3.61*	3.81	3.07*	3.79*	4.77**			3.43*
Coprophagy: No															

Legend: OR – The Odds ratio significance level is given by the number of asterisks: * - < 0.05; ** - < 0.01; *** - <0.001; AM - antimicrobial; TX - treatment; O. - owner; Prof. – profession; Health Prof. - healthcare professional; Cohabit.- cohabitant; AMP – ampicillin; AMC – amoxicillin-clavulanic acid; CEF – cephalothin; FOX – cephoxitin; CAZ – ceftazidime; CTX – cefotaxime; NAL – nalidixic acid; CIP – ciprofloxacin; GEN – gentamicin; NIT – nitrofurantoin; TET – tetracycline; STR – streptomycin; SXT – trimethoprim-sulfamethoxazol; CHL – chloramphenicol; KAN – kanamycin; IPM – imipenem; ATM – aztreonam.

Table 6. Risk markers for antimicrobial resistance of *E. coli* isolates from the multilevel multivariable final model.

Risk Markers	AMP OR	AMC OR	CEF OR	FOX OR	CAZ OR	CTX OR	NAL OR	CIP OR	GEN OR	TET OR	STR OR	SXT OR	CHL OR	KAN OR	ATM OR
Species Canine	5.16*														
Species Feline															
Gender Female	0.35*												0.28*		
Gender Male															
Reason: Check up		0.16*													
Reason: Illness															
Diet: Commercial														0.22*	
Diet: Mixed															
Quinolone Tx: Yes	6.02**		4.68*		16.78**	22.01**	13.51***	9.05**	4.73*	4.20**	4.55*	2.87*	4.79*		19.18***
Quinolone Tx: No															
O. Prof.: Health Prof.	3.95*	6.41*													
O. Prof.: Others															
Coprophagy: Yes	2.80*	10.35**		11.21*				3.12*			3.20*	3.73*			
Coprophagy: No															

Legend: OR – The Odds ratio significance leve lis given by the number of asterisks: * - < 0.05; ** - < 0.01; *** - <0.001; TX - treatment; O. - owner; Prof. – profession; Health Prof. - healthcare professional; Cohabit.- cohabitant; AMP – ampicillin; AMC – amoxicillin-clavulanic acid; CEF – cephalothin; FOX – cephoxitin; CAZ – ceftazidime; CTX – cefotaxime; NAL – nalidixic acid; CIP – ciprofloxacin; GEN – gentamicin; NIT – nitrofurantoin; TET – tetracycline; STR – streptomycin; SXT – trimethoprim-sulfamethoxazol; CHL – chloramphenicol; KAN – kanamycin; IPM – imipenem; ATM – aztreonam.

4. Discussion

Given the remarkable increase of AMR worldwide and the enormous difficulties and unsuccessful strategies to restrain its use, all efforts aiming to enlarge the knowledge in some of the many branches of this issue are of utmost importance. The present work was designed to assess the prevalence of AMR in enteric *E. coli* isolated from domestic cats and dogs in the region of Porto, Portugal, and to study potential risk markers for the presence of AMR in those isolates. This was accomplished with a GLMM, taking into account the multilevel structure of the data.

The proportions of AMR observed against ampicillin, cephalothin, tetracycline, streptomycin, trimethoprim-sulfamethoxazol, nalidixic acid, and ciprofloxacin were higher than previously reported (Costa et al., 2008; Murphy et al., 2009; Leonard et al., 2012). According to the categories proposed by EFSA (EFSA and ECDPC, 2013), 36.9% of the AM tested were assigned to the groups of high or very high resistance (Table 1). Interestingly none of the AM tested was classified in the extremely high category. In a study comprising fecal samples of 565 stray and 312 hospitalized dogs, Nam et al. (2010), reported generally higher AMR rates, however, this observation was already reached by those authors that, according to Korea Health Products Association data (about the amounts of antimicrobials usage in pets), believed to be related with the categories and elevated antimicrobials consumption rates in the country.

Considering that no antimicrobial was administered to the animals enrolled in the present study in the four months prior to sampling, our results corroborate the hypothesis that the reversibility of resistance in the absence of AM can be a slow process, probably due to compensatory evolution and cost-free resistance mechanisms (Andersson and Hughes, 2009). Although it could be stated that the Porto city area follows the urban trend to higher pet longevity, better veterinary care and widespread use of antibiotics in companion animal treatments, there are no evidences that such characteristics are in any way different from other studied areas. It has been demonstrated, however, that the Porto region suffers from a high level of environment contamination with antimicrobial resistance determinants (Novais et al., 2005; Simões et al., 2010; Flores et al., 2013), making plausible to assume that resistance acquisitions could be multifactorial (Martínez, 2012) and thus environment contamination exposure could also contribute for the high AMR rates exhibited.

Two of the variables influencing *E. coli* AMR deserve a special attention due to their relation with resistance to several antimicrobials. These risk markers are “prior quinolone treatment” and “coprophagic habits”. The discussion of these markers is undertaken subsequently.

By showing a coprophagic behavior the animal ingests gut microflora, including multidrug-resistant *E. coli* strains, from himself, which means a re-inoculation (autocoprophagy), or from other animals (allocoprophagy). Those strains, from autocoprophagy in particular, are expected to be straightforwardly adapted for prolonged colonization. Furthermore, feces from animals undergoing AM treatments, especially with poor oral bioavailability, may contain residual concentrations of the drug that are high enough to pressure the emergence and dissemination of AMR (Thaller et al., 2010; Toutain et al., 2010). Finally, several studies have already shown that there is a high level of horizontal gene transfer (HGT) within the intestine, and that its warm and nutrient rich environment makes it an ideal location for such phenomena (Lester et al., 2006; Hammerum and Heuer, 2009; Jakobsson et al., 2010).

Among the 15 studied antimicrobials, 12 revealed rates of resistance that were related to previous quinolone treatments. In fact, earlier quinolone exposure had already been pointed out as a risk marker for the emergence of AMR in *E. coli* isolated from food animals (Moniri and Dastehgoli, 2005) and humans (Cheong et al., 2001; McDonald et al., 2001; Lastours et al., 2010). This occurrence has been explained by the possible association of multiple antimicrobial resistance genes on mobile genetic elements (Moreno et al., 2008; Strahilevitz et al., 2009). Additionally, a strong association of plasmid-mediated quinolone resistance (PMQR) determinants with extended–spectrum–β–lactamases (ESBLs) or AmpC–type–β–lactamases has been reported (Moreno et al., 2008; Hammerum and Heuer, 2009; Strahilevitz et al., 2009; Rawat and Nair, 2010). These two types of resistance genes are often co-localized on the same plasmid, along with genetic determinants of other antimicrobial agents, such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Jacoby, 2009; Strahilevitz et al., 2009; Rawat and Nair, 2010; Zhao et al., 2010).

The significantly higher risk for ampicillin and amoxicillin-clavulanic acid resistances in pets whose owners are healthcare workers could be due to the combination of two driving forces. Firstly, since resistance genetic determinants for aminopenicillins circulate quite often among medical staff and facilities, pets are at increased risk to acquire antimicrobial-resistant *E. coli* from these owners (Hammerum and Heuer, 2009; Kalter et al., 2010; Martins da Costa et al., 2013). Secondly, the transfer of specific

resistance determinants to endogenous strains may be seriously enhanced by the recurrent exposure to the most prescribed oral antimicrobial drug in Portugal, amoxicillin-clavulanic acid (DGAV, 2011).

At the univariable level, the model used in the present study showed a clear and positive association between previous antimicrobial exposure and AMR. This was also reported by Moyaert et al. (2006), whose work with hospitalized animals retrieved frequencies of AMR quite similar to the ones from our study, which, in turn, included almost two thirds (62%) of patients with chronic conditions and, consequently, recurrently exposed to antimicrobial treatments. In fact, the animals reporting previous treatments in our study represented a group of risk in opposition to the "no previous antimicrobial treatment" group. A similar effect was observed at the variable "age" for the β-lactamics ampicillin, amoxicillin-clavulanic acid and cephalothin. So, younger and healthy animals carry less resistant *E. coli* strains, which may be linked with fewer opportunities of contact with antimicrobials. This, added to fewer cases of "coprophagic habits", is also a plausible explanation for the lower prevalence of antibiotic resistance in cats comparing with dogs that had 5.16 higher risk of carrying ampicillin-resistant *E. coli*.

Finally, at the univariable level, the different risk of contamination by multidrug-resistant *E. coli* in outdoor compared with indoor animals was not statistically significant. However, as found by Boothe (2012), being a male was considered a risk marker for resistance to ampicillin and chloramphenicol.

The limitations of the study are mainly related with the number of pets enrolled which was not calculated in advance because the purpose was to include the higher number of animals as possible given the time and the resources available. However, given that the selection process was random, the pets investigated represent the population of the hospital and an important factor of external validity was assured. The statistical analysis provides the significance necessary to assess the risk markers. Concerning the data collection, the questionnaires were performed by the same person and microbiological isolation, identification and antimicrobial resistance determination followed internal quality control procedures aimed to ensure reproducibility (consolidated methods performed by trained personnel) and accuracy (quality control of isolation and antimicrobial resistance media and internal control strains with known resistance pattern).

5. Conclusion

The present survey showed increased risk of AMR for enteric *E. coli* strains among the pets with record of previous quinolone treatments, which is in line with the results of several other reports in different animal species. The pets expressing a coprophagic behavior showed an important increase in the risk of AMR for enteric *E. coli* strains which points out the important role that the pet's owners shall play by educating their animals to control this behavior. Other markers like gender, species, reason for check-up were found statistically significant, but for a small number of antimicrobials, living room for further research of risk markers.

Conflict of interest statement

The authors declare that they have no conflicts of interest. markers.

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2.1.2. Paper II

PREVALENCE OF ANTIMICROBIAL RESISTANCE IN ENTERIC *ENTEROCOCCUS* SPP. FROM DOMESTIC PETS AND ASSESSMENT OF ASSOCIATED RISK FACTORS USING A GENERALIZED LINEAR MIXED MODEL

Leite-Martins, L., Mahú, M.I., Costa, A.L., Bessa, L., Vaz-Pires, P., Niza-Ribeiro, J., de Matos, A.J.F., da Costa, P.M.

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Antimicrobial resistance profiles of faecal enterococci from domestic dogs and cats and estimation of risk factors using a Generalized Linear Mixed Model

Liliana Leite-Martins[a,b*], Maria I. Mahú[b], Ana L. Costa[b], Lucinda J. Bessa[b,c], Paulo Vaz-Pires[d], João Niza-Ribeiro[e,f], Augusto J. F. de Matos[a,g], Paulo Martins da Costa[b,c]

[a] Abel Salazar Institute for the Biomedical Sciences (ICBAS), Department of Veterinary Clinics – UPVET, University of Porto, Porto, Portugal

[b] ICBAS, Laboratory of Microbiology and Food Technology, University of Porto, Porto, Portugal

[c] Interdisciplinary Center for Marine and Environmental Research (CIIMAR), University of Porto, Porto, Portugal

[d] ICBAS, Department of Aquatic Production, University of Porto, Porto, Portugal

[e] ICBAS, Department of Population Studies, University of Porto, Porto, Portugal

[f] ISPUP, Institute of Public Health, University of Porto, Porto, Portugal

[g] Animal Science and Study Centre / Food and Agrarian Sciences and Technologies Institute (CECA/ICETA), University of Porto, Portugal

Abstract

Antimicrobials have been falling to resistance and, as in human medicine, veterinarians are running out of options due to the high prevalence of infections found in companion animals caused by resistant microorganisms.

The present study aimed to determine the antimicrobial resistance profile of faecal enterococci isolated from pets, and investigate potential risk factors for antimicrobial resistance in those isolates associated with animals' characteristics, such as health status, individual habits, home environment, previous therapeutic events and owners' behaviour.

A total of 91 pets (74 dogs and 17 cats) were selected, using a systematic random procedure from the ordinary population of animals attending the Veterinary Hospital of Porto University, from September 2009 to May 2012. Animal owners, who allowed the collection of fecal samples from their pets, answered a questionnaire to collect information about the factors that could influence the AMR of fecal enterococci. Kanamycin Aesculin Azide Agar (Oxoid) (KAA) and Slanetz and Bartley Medium (Oxoid) (SB) were employed for enterococci isolation and disc diffusion method was used to determine antimicrobial susceptibility. The data were analyzed using a multilevel, univariable and multivariable generalized linear mixed model (GLMM).

From the 315 enterococci isolates obtained, 258 (81.9%) were obtained from dogs and the remaining 57 (18.1%) from cats. Sixty one per cent of the isolates were considered multi-drug resistant whereas only 9.2% were susceptible to all antimicrobials tested. Resistances found against tetracycline (67.0%), rifampicin (60.3%), azithromycin (58.4%), quinupristin/dalfopristin (54.0%) and erythromycin (53.0%), are causes for substantial concern. Previous quinolone treatments and coprophagic habits were the features more consistently associated with the presence of AMR to 3 and 7, respectively, out of the 9 antimicrobials assessed for risk factors.

The emergence and dissemination of AMR is a serious problem. Assessing the risk factors that determine the presence of drug-resistant bacteria in pets, a very close source of resistance determinants to humans, is crucial for the implementation of safer management procedures for pets and harmless selection of antimicrobial substances for the veterinary practitioners.

1. Introduction

During the last decades, the progress in veterinary medicine and the increased of social responsibilities for welfare and health of pets have conducted to a rise in pets longevity with a substantial augment in oncologic and geriatric pet patients, which have more propensity to chronic, debilitating and immunocompromising conditions and higher predisposition for needing antimicrobial (AM) treatments (da Costa *et al*., 2013). As in human medicine, AMs have been falling to resistance, and veterinarians are running out of options due to the high prevalence of infections found in companion animals caused by resistant microorganisms (Shaheen *et al.*, 2013; Prescott, 2014). In addition, the close contact between companion animals and humans builds up a unique and critical aspect related to antimicrobial resistance (AMR) that creates opportunities for inter-species transmission of (multidrug) resistant bacteria (Leite-Martins *et al.,* 2014). Therefore, small animal veterinarians must play an important role in the global approach for combating AMR. Monitoring AMR of pet isolates, as well as of the factors that regulate its emergence, are essential for assisting veterinary practitioners undertaking safer antimicrobial prescription. Without it the clinician tend to favor recent and with wider activity AMs.

Enterococci are common commensals of the intestinal microbiota of people and animals, however, they have emerged as one of the fourth most prevalent nosocomial pathogens worldwide, mostly because of their high resistance to antimicrobials, putative virulence traits and ability to form biofilm (Damborg *et al*., 2009; Gosh *et al*., 2011; Kwon *et al.,* 2012; Tremblay *et al*., 2013; Werner *et al*., 2013). Gosh *et al*. (2011) found that dogs, after being released from intensive care units and on antimicrobial treatment, harboured a large community of multidrug-resistant enterococci.

Thus, monitoring regularly the level of AMR in pathogens and normal flora has been recommended by the World Health Organization. Although some national and international surveillance programs on AMR have been established for food-producing animals (SENTRY, SCOPE, SWEDRES, SVARM, FAO, DANMAP and NARMS), pet animals are ordinarily not included in such programs (Gosh *et al*., 2011). The European Centre for Disease Prevention and Control, trough the Antimicrobial Resistance task force that encloses 53 countries, adopted a strategic action plan with tactical objectives and measures to protect specific key areas in order to restrain AMR spread (EFSA and ECDPC, 2013). However, surveillance programs should take into account the role of pets in AMR dissemination and analyze potential AMR-influencing factors, such as cohabitants

(humans and other pets) clinical antimicrobial records histories, domestic aggregate features and habits.

The aims of the present study were i) characterize AMR in faecal enterococci isolated from pets and ii) assess possible risk factors for that AMR associated with the health status and individual habits of the animals, some household characteristics as well as the presence and lifestyle of cohabitants (humans and other pets), especially in what concerns previous exposure to antimicrobials.

2. Materials and methods

2.1. Enrolment and sampling

The purpose of the study was reported to the eligible owners before requesting their collaboration. In order to collect a range of animals that could be representative of all pets visiting UPVET (Veterinary Hospital of Porto University, Portugal), a random systematic sampling procedure was adopted: only one pet a day was selected at a random hour, on Mondays or Tuesdays, from September 2009 to May 2012. Eligibility criteria required that the core animal have not taken any antimicrobial drugs (AM) during the 4 months preceding the selection. All participants were asked to sign a term of acceptance, to fill a questionnaire and to allow the collection of faecal samples (rectal swabs) from their pets. Approval was obtained from the Ethics Committee of the Abel Salazar Institute for the Biomedical Sciences, University of Porto.

2.2. Questionnaire

The questionnaire was designed with the aim of gathering information about possible risk factors for AMR acquisition by enterococci. Questionnaires used in previous studies about humans (Kalter *et al*., 2010; Lastours *et al*., 2010) and animals (Akwar *et al*., 2007; Ahmed *et al*., 2012; Boothe, 2012) were taken into consideration. The questionnaire included variables regarding individual characteristics of each animal like 1) species, 2) gender and 3) age and daily habits like 4) access to the outside environment (indoor habitat was assigned to those animals with very restricted access outdoor), 5) feeding (commercial food refers to the animals that were fed on strictly commercial dry or wet food) and 6) coprophagic habits (ingestion of feces, either their own or from other

animals), 7) previous systemic antimicrobial treatments with particular emphasis on 8) previous systemic quinolone treatments. Information on other potential risk factors like the 9) existence of cohabitant pets in the household, 10) previous antimicrobial treatments of human cohabitants, 11) the existence of "healthcare professionals" (human or veterinary hospitals, clinics or health centers workers) among the human cohabitants. The pets' health status was assessed through the 12) "reason for veterinary visit"; this information was given by the veterinary doctor based on the signs presented by the animal at the time of the examination.

2.3. Enterococci isolation

Faecal samples were collected by introducing saline-moistened swabs, with circular movements, into the rectum of each animal. The swabs were immediately immersed on Buffered Peptone Water (Oxoid, Basingstoke, UK) (BPW), transported to the laboratory and stored at room temperature for 30 minutes. From that suspension, an aliquot of 5 µl was streaked on Kanamycin Aesculin Azide Agar (Oxoid) (KAA) and Slanetz and Bartley Medium (Oxoid) (SB) for enterococci isolation. Plates were incubated at 37°C for 24 and 48 hours, respectively. After careful magnifier examination, two to five colonies with the typical appearance of enterococci were selected on the basis of colony size and morphology, trying to cover all morphologically different colonies (Leite-Martins *et al.,* 2014).

2.4. Antimicrobial susceptibility test

Antimicrobial susceptibility testing of each isolated enterococci was carried out by the disk diffusion assay, following guidelines provided by the "Clinical and Laboratory Standards Institute" (CLSI, 2012). A total of 12 antimicrobial agents were tested using antimicrobial impregnated disks (Oxoid) with: ampicillin (AMP, 25 µg), tetracycline (TET, 30 µg), rifampicin (RIF, 5 µg), gentamicin (GEN, 10 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (ERY, 15 µg), azithromycin (AZM, 15 µg), teicoplanin (TEC, 30 µg), vancomycin (VAN, 30 µg), quinupristin/dalfopristin (QD, 15 µg) and nitrofurantoin (NIT, 300 µg). The antimicrobial drugs were selected in order to include those regularly used in both human and veterinary medicine and to provide diversity by representing different antimicrobial classes. (Damborg *et al*., 2009; Jackson *et al*., 2009; EFSA and ECDPC, 2013). The interpretation of the inhibition zone length followed the

CLSI recommendations and breakpoints for enterococci. (CLSI, 2012). Multidrug-resistant bacteria were considered according to previous definition (Magiorakos *et al.,* 2011).

2.5. Data analysis

The prevalence of AMR for each AM was calculated by dividing the number of the respective AM-resistant enterococci isolates by the total number of enterococci tested. The potential risk factors obtained from the questionnaire were transformed into categorical variables as follows: dichotomous variables - species (canine, feline), gender (male, female), reason for veterinary visit (routine check-up, illness signs), habitat type (indoor, mixed), food type (commercial, mixed), previous quinolone treatments (yes, no), health professionals owners (yes, no), owners submitted to previous antimicrobial treatments (yes, no), cohabitant pets (yes, no), coprophagy habits (yes, no). The historical record of the animal having previous antimicrobial treatments was transformed into three categories: “none”, “just one” and “two or more” treatments; the age was also transformed in three categories: “young” animals with less than 2 years of age, “adult” animals between 2 and 10 years and “old” animals with more than 10 years. The result of AMR is the outcome of the models and was dichotomized in resistant or sensitive; intermediate results were categorized as sensitive. According to the European Food Safety Authority criteria, each antimicrobial was further classified into one of the following categories of prevalence of AMR: extremely high: > 70%; very high: 50-70%; high: 20-50%; moderate: 10-20%; low: 1-10%; very low: 0.1-1% and rare: <0.1% (EFSA and ECDPC, 2013).

A descriptive analysis of both AMR prevalence and frequencies of risk factors was performed. A Multilevel Generalized Linear Mixed Model (GLMM) was used to analyze the potential risk factors for AMR and to assess the strength of their associations.

The logit link function was used to model the probability of occurrence of resistance to an antibiotic. To take into account the multilevel structure of the data in which more than one enterococci strain (*i*) was isolated from each animal (*j*), a two level structure in the data was assumed in which enterococci strains (first level) were nested within the animal from which they were isolated (second level).

The data were modeled in the following way:

$Y = \begin{cases} 0\ (no\ AMR) \\ 1\ (AMR) \end{cases}$ Where Y is the response variable.

Pr(Y) = pij, i = 1, …, 315 and j = 1, ..., 91.

The generic model used the following equation: $logit(p_{ij}) = a + c_j + \beta\ \ animal\ variables_j$ The model, the animal (the pet) was allowed to be random. The second level random effect is given by $c_j \sim N(0,\sigma 2)$ where σ2 is the variance of the random effects at the animal level.

The basic multivariable multilevel model was as follows:

$$logit(p_{ij}) = a + c_j + \beta_1 Species_j + \beta_2 Age_j + \beta_3 Gender_j + \beta_4 Reason\ of\ visit_j + \beta_5 Habitat_j + \beta_6 Diet_j + \beta_7 Number\ AM\ treatments_j + \beta_8 Previous\ Quinolones\ treatments_j + \beta_9 Owner's\ profession_j + \beta_{10} Owner's\ AM\ treatments_j + \beta_{11} Cohabitants\ Pets_j + \beta_{12} Coprophagy\ habits_j$$

Variables codes are presented in Tables 3 to 5.

A three step procedure was taken as follows: firstly, a univariable multilevel GLMM analysis was conducted to assess the individual relationship between each potential risk factor and the presence of AMR; a second step was performed to conduct a multivariable multilevel GLMM analysis with all the variables that had a $p < 0.15$ in the previous analyses followed by a manual backward and forward procedure to obtain a final model where each factor effect was adjusted for the remaining factors. Only factors with a $p < 0.05$ were retained in the final model. The data were analyzed using the procedure GEE in the SPSS Software V. 21.0 (IBM SPSS statistical 21 package, IBM Corporation, NY).

3. Results

A total of 91 pets (74 dogs and 17 cats), one per household, were enrolled in the present study. Out of the 315 isolates of enterococci, 258 (81.9%) were obtained from

dogs and the remaining 57 (18.1%) from cats. The number of isolates from each animal ranged from 2 to 5 with an average of 3.46 per pet.

3.1. Presence of multidrug-resistant enterococci

Only 9.2% of the isolates were susceptible to all antimicrobials tested. According to previous definition of multidrug resistance (Magiorakos *et al*., 2011), the majority (61.9%) of enterococci isolates was considered multidrug-resistant; 50% showed resistance to three AM and 75% were resistant to six AM. Two isolates (0.6%) and three isolates (1%) showed resistance towards 9 and 8 AM, respectively. The histogram of these isolates displayed by the absolute number of resistances per isolate suggests the existence of two sub-populations of enterococci and is shown in Fig. 1: one group with less than three resistances and a second group with more than 5 resistances.

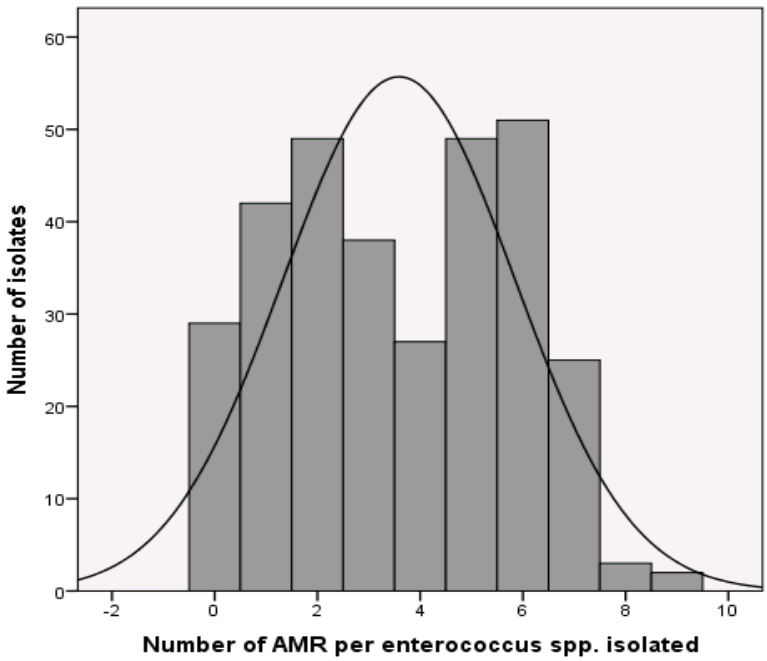

Fig. 1. Incidence of antimicrobial resistance in isolated *enterococci* (n=315).

3.2. Antimicrobial resistance prevalence

The prevalence of AMR per antimicrobial varied from 1.0% found in vancomycin up to 67.0% for tetracycline. After categorization according to the EFSA (EFSA and ECDPC, 2013) recommendations, 41.7% of the AM tested were at the very high resistance category and a similar proportion of antimicrobials showed a low level

resistance. Again a bimodal pattern seems to occur. The high and moderate resistance categories harbored 8.3% of the tested AM each, as displayed in Table 1.

Table 1. Categorization of antimicrobial (AM) resistance profile in the isolated *enterococci* according to the EFSA risk categories for prevalence of resistances and proportion of AM in each category.

EFSA Risk Categories	AM	Prevalence of resistance	C.I.	Frequency of AM in each category	C.I.
Extremely High		0.0	-	0.0	-
Very High	TET	67.0	0.046	41.7	0.049
	RIF	60.3	0.048		
	AZM	58.4	0.049		
	QD	54.0	0.049		
	ERI	53.0	0.049		
High	CIP	29.5	0.045	8.3	0.027
Moderate	AMP	12.1	0.032	8.3	0.027
Low	NIT	9.2	0.028	41.7	0.049
	GEN	6.3	0.024		
	CHL	6.3	0.024		
	TEC	2.2	0.014		
	VAN	1.0	0.010		
Very Low		0.0	-	0.0	-
Rare		0.0	-	0.0	-

Legend: AM – antimicrobial agent; C.I. – confidence interval; AMP – ampicillin; TET – tetracycline; RIF - rifampicin; GEN – gentamicin; CHL - chloramphenicol; CIP – ciprofloxacin; ERI - erythromycin; AZM - azithromycin; TEC – teicoplanin; VAN - vancomycin; QD - quinupristin/dalfopristin; NIT – nitrofurantoin. Values are expressed in percentages.

3.3. Descriptive analysis of risk factors and pets

The allocation of pets and percentage of isolates into the different categories of potential risk factors is presented in Table 2. The proportions of antimicrobial resistant strains for each potential risk factor are displayed in Table 3. The AMR proportions were calculated based in all enterococci isolates (n=315). The overall data displayed lower resistance numbers in potential risk factors for AMR labeled into the moderate and low resistance level categories (EFSA and ECDPC, 2013).

Table 2. Distribution of pets (n=91) among potential risk factor categories and *enterococci* isolates in percentage of the total number of isolates (n=315).

Risk factor	Category	Dogs	% isolates	Cats	% isolates	Total Pets	Isolates (%)
Species		74		17		91	100
Age	< 2 years	22	24.1	8	8.3	30	32.4
	2 - 10 years	30	34.3	8	8.9	38	43.2
	> 10 years	22	23.5	1	1.0	23	24.4
Gender	Female	43	48.6	10	10.2	53	58.7
	Male	31	33.3	7	7.9	38	41.3
Reason for veterinary visit	Check up	23	24.8	11	11.7	34	36.5
	Illness	51	57.1	6	6.3	57	63.5
Habitat Type	Indoor	7	7.6	13	13.7	20	21.3
	Mixed	67	74.3	4	4.4	71	78.7
Food Type	Commercial	23	26.3	7	6.7	30	33.0
	Mixed	51	55.6	10	11.4	61	67.0
Animal Antimicrobial Treatments	None	21	21.0	13	13.7	34	34.6
	Just One	15	17.5	2	1.9	17	19.4
	Two or more	38	43.5	2	2.5	40	46.0
Animal Quinolone Treatments	Yes	27	30.2	2	2.5	29	32.7
	No	47	51.7	15	15.6	62	67.3
Owners Health Professionals	Yes	15	15.9	5	4.8	20	20.6
	No	59	66.0	12	13.3	71	79.4
Owners Antimicrobial Treatments	Yes	35	42.9	4	4.4	39	47.3
	No	39	39.0	13	13.7	52	52.7
Cohabitant Pets	Yes	33	40.0	13	13.7	46	53.7
	No	41	41.9	4	4.4	45	46.3
Coprophagy Habits	Yes	29	32.7	4	4.4	33	37.1
	No	45	49.2	13	13.7	58	62.9

Table 3. Antimicrobial resistant isolates distribution by potential risk factor categories (n=315).

Variables	Category	% of resistant isolates / antimicrobial											
		AMP	TET	RIF	GEN	CHL	CIP	ERI	AZM	TEC	VAN	QD	NIT
Species	Canine	12.1	55.9	47.9	6.0	5.4	25.1	44.8	49.8	2.2	1.0	44.1	7.9
	Feline	0.0	11.1	12.4	0.3	1.0	4.4	8.3	8.6	0.0	0.0	9.8	1.3
Age	< 2 years	1.9	17.8	16.8	1.6	3.5	4.8	14.6	14.3	0.6	0.6	15.6	2.2
	2 - 10 years	3.5	29.8	29.5	2.5	0.3	13.3	22.2	28.9	1.0	0.0	25.7	3.8
	> 10 years	6.7	19.4	14.0	2.2	2.5	11.4	16.2	15.2	0.6	0.3	12.7	3.2
Gender	Female	7.3	39.4	35.6	2.5	6.0	15.9	32.7	35.2	1.3	0.6	28.9	6.3
	Male	4.8	27.6	24.8	3.8	0.3	13.7	20.3	23.2	1.0	0.3	25.1	2.9
Reason for veterinary visit	Check up	3.8	22.9	19.4	2.9	1.9	6.3	18.4	18.4	0.3	0.6	18.4	4.1
	Ilness	8.3	44.1	41.0	3.5	4.4	23.2	34.6	40.0	1.9	0.3	35.6	5.1
Habitat Type	Indoor	1.0	14.3	13.7	0.6	1.0	6.7	10.5	10.5	1.6	0.3	9.5	1.6
	Mixed	11.1	52.7	46.7	5.7	5.4	22.9	42.5	47.9	0.6	0.6	44.4	7.6
Food Type	Comercial	5.4	23.2	18.4	3.2	2.5	12.1	18.7	19.7	0.6	0.3	16.8	4.4
	Mixed	6.7	43.8	41.9	3.2	3.8	17.5	34.3	38.7	1.6	0.6	37.1	4.8
Animal Antimicrobial Treatments	None	0.3	21.0	20.3	2.2	1.3	5.4	14.0	16.5	0.3	0.6	15.9	2.5
	Just One	1.6	14.3	12.4	1.3	2.5	5.4	10.2	11.1	1.3	0.0	11.7	1.9
	Two or more	10.2	31.7	27.6	2.9	2.5	18.7	28.9	30.8	0.6	0.3	26.3	4.8
Animal Quinolone Treatments	Yes	5.4	25.7	20.0	2.9	5.1	18.1	22.5	23.2	1.3	0.6	20.0	3.2
	No	6.7	41.3	40.3	3.5	1.3	11.4	30.5	35.2	1.0	0.3	34.0	6.0
Owners Health Professionals	Yes	3.2	12.7	10.5	1.3	1.6	5.7	11.4	11.4	0.0	0.0	10.5	2.9
	No	8.9	54.3	49.8	5.1	4.8	23.8	41.6	47.0	2.2	1.0	43.5	6.3
Owners Antimicrobial Treatments	Yes	4.4	32.1	27.3	4.1	4.4	12.7	27.6	30.5	2.2	0.6	26.3	4.4
	No	7.6	34.9	33.0	2.2	1.9	16.8	25.4	27.9	0.0	0.3	27.6	4.8
Cohabitant Pets	Yes	5.4	38.4	32.7	4.8	6.3	16.8	30.5	33.7	0.6	0.6	27.6	4.8
	No	6.7	28.6	27.6	1.6	0.0	12.7	22.5	24.8	1.6	0.3	26.3	4.4
Coprophagy Habits	Yes	7.0	29.8	25.7	4.1	5.4	15.6	25.7	27.0	0.0	0.3	22.2	4.4
	No	5.1	37.1	34.6	2.2	1.0	14.0	27.3	31.4	2.2	0.6	31.7	4.8

Legend: AMP – ampicillin; TET – tetracycline; RIF - rifampicin; GEN – gentamicin; CHL - chloramphenicol; CIP – ciprofloxacin; ERI - erythromycin; AZM - azithromycin; TEC – teicoplanin; VAN - vancomycin; QD - quinupristin/dalfopristin; NIT – nitrofurantoin.

3.4. Results of the multilevel univariable analysis

Table 4 displays the results of the multilevel univariable analysis. Only variables and categories with Odds Ratio (OR) lower than 0.15 are presented. The factors demonstrating a significant ($p < 0.05$) increased risk of resistance were: being a female (for chloramphenicol); living indoor (for teicoplanin); having received previous quinolone treatments (for tetracycline, chloramphenicol, ciprofloxacin, erythromycin and azithromycin); being owned by persons who have already done antimicrobial treatments (for chloramphenicol and azithromycin) and having coprophagic habits (for tetracycline, rifampicin, gentamycin, chloramphenicol, ciprofloxacin, erythromycin and azithromycin). Protective factors ($p < 0.05$) were: being younger than 2 years (for tetracycline, ciprofloxacin and azithromycin), younger than 10 years old (for ampicillin and erythromycin) or having between 2 and 10 years old (for chloramphenicol). Regarding the reason to visit the veterinary, coming for check-up appears also a protective factor (for rifampicin, ciprofloxacin and azithromycin); as well as have never taken antimicrobial treatments (for ampicillin, ciprofloxacin, erythromycin and azithromycin). Only quinupristin/dalfopristin, vancomycin and nitrofurantoin resistances failed to be significantly associated with any potential risk factor and by this reason these antimicrobials were excluded from the subsequent multilevel multivariable analysis.

3.5. Results of the multilevel multivariable analysis

The variables included in the multilevel multivariable model were selected from the previous univariable analysis, when a $p < 0.15$ was considered. The final model was obtained from a multilevel multivariate analysis after manual backward and forward variable selection and resulted in a robust model. Only the variables that, after adjustment for all the other variables remained significant at $p < 0.05$, were kept in this model (Table 5). The final model showed that the factors demonstrating a significant ($p < 0.05$) increased risk of resistance were: being a female (for chloramphenicol); living indoor (for teicoplanin); having received one previous antimicrobial treatment (for chloramphenicol), having received previous quinolone treatments (for chloramphenicol, ciprofloxacin and azithromycin) and having coprophagic habits (for tetracycline, rifampicin, gentamycin, chloramphenicol, ciprofloxacin, erythromycin and azithromycin). Protective factors were: being a female (for gentamycin) and have not been treated with any antimicrobial (for ampicillin and erythromycin).

Table 4. Risk factors for antimicrobial resistance of *enterococci* isolates displayed from the univariable multilevel analysis.

Risk Factors	AMP	TET	RIF	GEN	CHL	CIP	ERI	AZM	TEC
	OR	OR	OR	OR	OR	OR	OR	OR	OR
Species Canine								1.73	
Species Feline									
Age: < 2 years	0.11**	0.22*	0.81		1.04	0.11**	0.42**	0.48*	
Age: 2 - 10 years	0.22*	0.54	1.62		0.06*	0.43	0.54*	1.22	
Age: > 10 years									
Gender Female				0.44	14.77*				
Gender Male									
Reason: Check up			0.62*			0.26*		0.60*	
Reason: Ilness									
Habitat: Indoor	0.27						0.46	0.62	9.92**
Habitat: Mixed									
Food: Commercial				2.14					
Food: Mixed									
AM Tx: None	0.04**				0.65	0.21**	0.40**	0.45**	0.66
AM Tx: One	0.18				2.59	0.42	0.66	0.67	5.02
AM Tx: 2 or +									
Quinolone Tx: Yes		2.93*		4.50	9.56***	9.47***	2.68***	2.21**	
Quinolone Tx: No									
O. Prof.: Health Prof.			0.61						
O. Prof.: Others									
O. AM Tx: Yes					2.77*		1.51	1.61*	
O. AM Tx: No									
Cohabit. Pets: Yes			2.06				1.39	1.57	
Cohabit. Pets: No									
Coprophagy: Yes	3.09	3.83**	1.84*	3.96**	11.05***	3.64**	2.93***	2.66***	
Coprophagy: No									

Legend: OR – Odds ratio; * – p value (* - <0.05; ** - <0.01; *** - <0.001); AM - antimicrobial; TX - treatment; O. - owner; Prof. - professional; Cohabit.- cohabitant; AMP – ampicillin; TET – tetracycline; RIF - rifampicin; GEN – gentamicin; CHL - chloramphenicol; CIP – ciprofloxacin; ERI - erythromycin; AZM - azithromycin; TEC – teicoplanin.

Table 5. Risk factors for antimicrobial resistance of *enterococci* isolates displayed from the multilevel multivariable final model.

Risk Factors	AMP	TET	RIF	GEN	CHL	CIP	ERI	AZM	TEC
	OR	OR	OR	OR	OR	OR	OR	OR	OR
Gender Female				0.35*	16.04*				
Gender Male									
Habitat: Indoor									10.33*
Habitat: Mixed									
AM Tx: None	0.06*				13.15		0.36*		
AM Tx: One	0.26				9.66*		0.72		
AM Tx: 2 or +									
Quinolone Tx: Yes					22.72**	8.39***		1.94***	
Quinolone Tx: No									
Coprophagy: Yes		3.01*	2.56	3.96**	10.46*	2.75*	3.82**	2.42***	
Coprophagy: No									
Risk Factor Patterns		A	A			B		B	

Legend: OR – Odds ratio; * – p value (* - <0.05; ** - <0.01; *** - <0.001); AM - antimicrobial; TX - treatment; O. - owner; Prof. - professional; Cohabit.- cohabitant; AMP – ampicillin; TET – tetracycline; RIF - rifampicin; GEN – gentamicin; CHL - chloramphenicol; CIP – ciprofloxacin; ERI - erythromycin; AZM - azithromycin; TEC – teicoplanin.

4. Discussion

In this study, we aimed to establish the prevalence of AMR against 12 antimicrobials in 315 faecal enterococci isolated from dogs and cats attending UPVET. The use of a GLMM multilevel model to analyse the data allowed the identification of risk factors significantly associated with the presence of AMR within the sampled population.

A random systematic sampling procedure was adopted, in order to collect a range of animals representative of all pets visiting the hospital. After comparing the factors species, age, sex and reason for the visit from our sample with that from the UPVET population, it was concluded that the population of pets enrolled, resembles that of the pets attending the UPVET hospital. The existence of two enterococci sub-populations was suggested through the histogram exhibiting the incidence of the AMR in total enterococci

isolated (Figure 1), one categorized at the very high resistance level and other at the low resistance level (EFSA and ECDPC, 2013), reinforcing the bimodal pattern that was supposed to occur.

Enterococci infections are unusual in dogs and cats. However, the extent of AMR in enterococci from companion animals should be monitored to provide baseline information and to fully assess the role that these animals could have as reservoirs of resistant bacteria and their potential impact on humans and on the environment. A considerable proportion of the enterococci displayed resistance to tetracyclines, macrolides, clindamycin, rifampin and fluoroquinolones. It seems likely that these resistances have emerged among enterococci that were colonizing animals to which antimicrobials were given for other reasons. Antimicrobial therapy affects not only the target pathogen but also commensal inhabitants of the host, namely those from the gut microbiota (Jackobsson *et al*., 2010). The extent of the impact on non-target microbial populations depends on the particular drug used, on its mode of action and on the degree of resistance in the community (Jernberg *et al*., 2010). Colonizing bacteria may actually be more capable of developing resistance because they coexist with multiple other bacterial species and therefore have access to their resistance genes.

Some studies aimed to monitor bacterial susceptibility to antimicrobials among faecal enterococci isolated from pets were previous performed (Poeta *et al.,* 2006; Damborg *et al*., 2009; Jackon *et al*., 2009; Ghosh *et al*., 2011; Hamilton *et al*., 2013). AMR frequencies found in the present survey were overall higher when compared with the above studies, with the exception for Ghosh *et al*., (2011), probably because intensive care unit animals under antimicrobial treatment were sampled. The work from the North of Portugal (Poeta *et al.*, 2006) was realized just with healthy animals, a possible reason for lower AMR frequencies when compared with the ones we obtained. The Porto region is known to have a high level of antimicrobial environment contamination (Flores *et al.,* 2013), a potential contribution for our pool of animals to have higher AMR resistance rates when compared with the other previous comparable studies.

The multilevel univariable model (Table 4) displayed several risk factors. The final form obtained from the statistical analysis, using a multilevel multivariable model (Table 5), resulted in an important reduction in the number of significant risk factors ($p < 0.05$), when compared with the initial multilevel invariable model. Strong risk factors as “Previous Quinolone Treatments” and “Coprophagy habits” were practically maintained, promoting AMR in 3 and 7 drugs through the multivariable final model, respectively. Other factors such as “Age”, “Reason for the Veterinary Visit” or “Owners’ AM Tx” lost their strength.

However, the fact that these risk factors were significant at the univariable model, may suggest that the design of further risk factor studies, in the future, should take into consideration these results to clarify their importance.

Two distinct patterns of risk factors could be proposed (Table 5): pattern A) which associates coprophagic behaviors with AMR to tetracycline and rifampicin, and pattern B) that connects animals with coprophagic habits and previously treated with quinolones to be at a higher risk for harboring isolates resistant to ciprofloxacin and azithromycin.

The direct ingestion of resistant bacteria is a way to acquire AMR (Lastours *et al*., 2010). Coprophagy, which comprises the ingestion of intestine inhabitants straightforwardly adapted for prolonged colonization (mainly in autocoprophagy), can be seen as a form of amplifying the variability of bacterial cells as well as resistance determinants. Several studies have already shown that the transfer of resistance genes can occur at a high level within the intestine, (Lester *et al*., 2006; Jackobsson *et al*., 2010; Jernberg *et al*., 2010), which is the ideal location for such phenomena to occur, since it provides a warm and nutrient-rich environment with large numbers of bacterial cells potentially able to develop resistance mechanisms and exchange resistance determinants. Enterococci have natural gene transfer mechanisms that allow the acquisition of multiple resistances (Jackson *et al*., 2009). Furthermore, since some faeces may contain extremely high concentrations of antimicrobials, especially of drugs with poor oral bioavailability, eating faeces may lead to drug transfer between animals (allocoprophagy) or within the same animal (autocoprophagy), enhancing the emergence and dissemination of AMR (Thaller *et al*., 2010; Toutain *et al*., 2010).

Pets' intestinal colonization by ciprofloxacin and/or azithromycin-resistant enterococci seems to be highly influenced by pets' "Previous Quinolone Treatments". Some authors (Yasufuku, 2011; Dalhff, 2012; Lee, 2013) had already found a significant positive statistical correlation between the previous use of fluoroquinolones and enterococci resistance to quinolones, in humans. As resistance to macrolides (azithromycin), streptogramins and tetracyclines can be co-selected by fluoroquinolone agents (Poole, 2005; Zechini and Versace, 2009; Dalhff, 2012), this could explain the observed relation between prior quinolone treatments and azithromycin resistance.

Previous studies displayed similarities between human and pet enterococci isolates (Damgorg *et al*., 2009; Kwon *et al*., 2012; Tremblay *et al.*, 2013), however our data failed to find any significance for animals belonging to health professionals or people

already exposed to antimicrobial treatments; this result may be explained by the low number of animals analysed in those groups (Table 2).

Animals younger than two years old were at lower risk of being colonized by enterococci resistant to ampicillin, tetracycline, ciprofloxacin, erythromycin and azithromycin. This could be explained through the less time and few opportunities that younger animals have to had antimicrobial treatments or to have contacted resistance genetic determinants from the pool of ambient contamination, a well-known source of AMR determinants acquisition (Andersson and Hughes, 2010; Martínez, 2012; Flores *et al.,* 2013),.

Regular monitoring of the level of AMR in pathogens and normal flora has been recommended by the World Health Organization and pets, sharing so many aspects of their lifes with the owners, should have an important place on that.

Studies in this field are needed in order to understand the mechanisms involved in the emergence, spread, maintenance and evolution of antimicrobial resistance. The present data may just pretend to alert and reinforce the attention to one small piece of the enormous AMR puzzle.

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2.2. HOUSEHOLD ANTIMICROBIAL RESISTANCE SHARE AND SPREAD

2.2.1. Paper III

COMMON PHENOTYPIC AND GENOTYPIC ANTIMICROBIAL RESISTANCE PATTERNS FOUND IN A CASE STUDY OF MULTIRESISTANT *E. COLI* FROM COHABITANT PETS, HUMANS, AND HOUSEHOLD SURFACES

Martins, L.R., Pina, S.M., Simões, R.L., de Matos, A.J., Rodrigues, P., da Costa, P.M.

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INTERNATIONAL PERSPECTIVES

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Common Phenotypic and Genotypic Antimicrobial Resistance Patterns Found in a Case Study of Multiresistant *E. coli* From Cohabitant Pets, Humans, and Household Surfaces

Although most of the information presented in the Journal refers to situations within the United States, environmental health and protection know no boundaries. The Journal periodically runs International Perspectives to ensure that issues relevant to our international membership, representing over 20 countries worldwide, are addressed. Our goal is to raise diverse issues of interest to all our readers, irrespective of origin.

Liliana Raquel Leite Martins, DVM
Susana Maria Rocha Pina, PhD
Romeo Luís Rocha Simões, DVM
Augusto José Ferreira de Matos, DVM, PhD
Pedro Rodrigues, PhD
Paulo Martins Rodrigues da Costa, DVM, PhD
Institute for the Biomedical Sciences Abel Salazar
Porto University

Abstract **The objective of the study described in this article was to characterize the antimicrobial resistance profiles among *E. coli* strains isolated from cohabitant pets and humans, evaluating the concurrent colonization of pets, owners, and home surfaces by bacteria carrying the same antimicrobial-resistant genes. The authors also intended to assess whether household surfaces and objects could contribute to the within-household antimicrobial-resistant gene diffusion between human and animal cohabitants. A total of 124 *E. coli* strains were isolated displaying 24 different phenotypic patterns with a remarkable percentage of multiresistant ones. The same resistance patterns were isolated from the dog's urine, mouth, the laundry floor, the refrigerator door, and the dog's food bowl. Some other multiresistant phenotypes, as long as resistant genes, were found repeatedly in different inhabitants and surfaces of the house. Direct, close contact between all the cohabitants and the touch of contaminated household surfaces and objects could be an explanation for these observations.**

Introduction

The use of antimicrobial drugs induces the increase of antimicrobial resistance, not just in the pathogenic bacteria but also in the endogenous commensal flora (Berge, Moore, & Sischo, 2006; Costa et al., 2008a; Dancer, 2004; Goossens, 2009; Goossens, Ferech, Stichele, & Elseviers, 2005; van den Bogaard & Stobberingh, 2000). During the last decade, an awareness has been increasing of the potential problems that selection for antimicrobially resistant bacteria among companion animals may cause on human health, due to the increasing utilization of the same antimicrobial substances in human medicine and to the close contact between pets and their human cohabitants (Guardabassi, Loeber, & Jacobson, 2004; Guardabassi, Schwarz, & Lloyd, 2004; Moyaert, de Graef, Haesebrouck, & Decostere, 2006; Schwarz, Kehrenberg, & Walsh, 2001). The growing number of household pets and their increasing health care standards has led to an augmented number of geriatric animals accompanied by extensive medical histories including antimicrobial drug administration and longer contact with owners increasing both the risk of antimicrobial resistance emergence and interspecies clonal spread.

The spread of antimicrobial-resistant bacteria can occur directly, by skin-to-skin contact and contact with bacteria-containing material (e.g. saliva, feces), or indirectly via the household environment (Guardabassi, Loeber, & Jacobson, 2004; Schwarz et al., 2001). When reaching the new host, resistant bacteria can either colonize and infect, or remain in that particular environment for only a very short period of time. During this period, the resistant bacteria can not only spread their resistance genes to other bacteria residing in the new host (commensals or pathogens), but also accept resistance genes from other bacteria (Livermore, 2003; Schwarz et al., 2001).

E. coli has a great ecological value in the assessment of resistance spreading not only because it plays an important role as acceptor and donor of transmissible drug-

TABLE **1**

Primers Used for Detection of Genes Encoding Antimicrobial Resistance in *E. coli* Isolates

Target Gene	Primer	Nucleotide Sequence (5'–3')	Size (Base Pair)	Reference
ampC	ampC-F	CCCCGCTTATAGAGCAACAA	634	Mendonça et al. (2007)
	ampC-R	TCAATGGTCGACTTCACACC		
bla_{TEM}	TEM-F	ATTCTTGAAGACGAAAGGGC	1150	Costa et al. (2008a); Sáenz et al. (2004)
	TEM-R	ACGCTCAGTGGAACGAAAAC		
bla_{OXA}	OXA1F	ACACAATACATATCAACTTCGC	813	Costa et al. (2008a); Sáenz et al. (2004)
	OXA1R	AGTGTGTTTAGAATGGTGATC		
bla_{SHV}	SHV-F	CACTCAAGGATGTATTGTG	885	Costa et al. (2008a); Sáenz et al. (2004)
	SHV-R	TTAGCGTTGCCAGTGCTCG		
bla_{CTX-M}	CTX-F	TTTGCGATGTGCAGTACCAGTAA	543	Mendonça et al. (2007)
	CTX-R	CGATATCGTTGGTGGTGCCATA		
$bla_{CTX-M-15}$	CTX15F	AGAATAAGGAATCCCATGGTT	875	Mendonça et al. (2007)
	CTX15R	ACCGTCGGTGACGATTTTAG		
aadA	AadA-F	GCAGCGCAATGACATTCTTG	282	Sáenz et al. (2004)
	AadA-R	ATCCTTCGGCGCGATTTTG		
strA	StrA-F	CTTGGTGATAACGGCAATTC	548	Srinivasan et al. (2007)
	StrA-R	CCAATCGCAGATAGAAGGC		
strB	StraB-F	ATCGTCAAGGGATTGAAACC	509	Srinivasan et al. (2007)
	StraB-R	GGATCGTAGAACATATTGGC		
gyrA	GyrA-F	TACACCGGTCAACATTGAGG	648	Costa et al. (2008a)
	GyrA-R	TTAATGATTGCCGCCGTCGG		
parC	ParC-F	AAACCTGTTCAGCGCCGCATT	395	Costa et al. (2008a)
	ParC-R	GTGGTGCCGTTAAGCAAA		
tetA	TetA-F	GTAATTCTGAGCACTGTCGC	937	Costa et al. (2008a)
	TetA-R	CTGTCCTGGACAACATTGCTT		
tetB	TetB-F	CTCAGTATTCCAAGCCTTTG	416	Costa et al. (2008a)
	TetB-R	CTAAGCACTTGTCTCCTGTT		
cml	CML-F	CCGCCACGGTGTTGTTGTTATC	698	Sidjabat et al. (2006)
	CML-R	CACCTTGCCTGCCCATCATTAG		
flo	FLO-F	TATCTCCCTGTCGTTCCAG	399	Sidjabat et al. (2006)
	FLO-R	AGAACTCGCCGATCAATG		
catA	M62822	AGTTGCTCAATGTACCTATAACC	547	Maynard et al. (2007)
	M62822	TTGTAATTCATTAAGCATTCTGCC		
sul1	Sul1-F	TGGTGACGGTGTTCGGCATTC	789	Costa et al. (2008a); Sáenz et al. (2004)
	Sul1-R	GCGAGGGTTTCCGAGAAGGTG		
sul2	Sul2-F	CGGCATCGTCAACATAACC	722	Costa et al. (2008a); Sáenz et al. (2004)
	Sul2-R	GTGTGCGGATGAAGTCAG		

resistant genes transferable to pathogenic bacteria (Sáenz et al., 2004; van den Bogaard & Stobberingh, 2000), but also because it is commonly found in the intestinal tract of humans and animals (Costa et al., 2008a). *E. coli* can also be implicated in various intestinal and extraintestinal diseases (Johnson, Owens, Gajewski, & Clabots, 2008; Johnson, Stell, & Delavari, 2001). Usually the host's own fecal flora is the immediate source of the extraintestinal pathogenic *E. coli* strains. The external reservoirs from which the hosts initially acquire such strains and the relevant transmission mechanisms, however, are poorly understood (Johnson et al., 2008).

The aim of our study was to characterize phenotypically and genetically the antimicrobial resistance profiles among *E. coli* strains isolated from cohabitant pets and humans, considering the concurrent colonization of pets, owners, and home surfaces by bacteria with the same resistance patterns and carrying the same antimicrobial-resistant genes.

Methods

Enrollment and Sampling

Case selection emerged from the universe of clients of the Institute of Biomedical Sciences Abel Salazar Companion Animals Veterinary Clinic (Porto University, Portugal). The participants were chosen taking into account that both the man and the dog had already been administered several antimicrobial treatments and that the dog was recently diagnosed with a recurrent urinary tract infection. A formal consent was signed and a complete questionnaire, including environment, human and veterinary medical records with antibiotic usage by themselves, family members, and their pets was completed.

A dog's oral swab and cystocentesis for urine collection were carried out immediately. Cystocentesis was performed by aseptic technique: prepubic hair was clipped and the skin was cleaned and disinfected with alcohol and clorhexidine before the insertion of a needle connected to a 10 mL syringe to collect urine directly from the dog's bladder. Fecal samples of the two adults (male and female owners), of their two-year-old grandchild (daily cohabitant), and of the household cat and dog were delivered the next morning.

Simultaneously, the following household environmental swabs were collected: two from light switches, one from the refrigerator door handle, two from door knobs, two from the dog's food and water bowls, one from the laundry floor, and one from each owner's hands.

E. coli Isolation

After reception at the laboratory, fecal samples were immediately diluted 1:10 in saline buffer and stored at room temperature for one hour. From this initial suspension, an aliquot of

TABLE **2**

Number of Antimicrobial Resistance Patterns in *E. coli* Isolates From Pets, Human Cohabitants, and Household Environment

Antimicrobial[a] Resistance Pattern	Pets				Human Cohabitants			Household Environment		
	Dog Feces	Dog Urine	Dog Mouth	Cat Feces	Male Feces	Female Feces	Child Feces	Laundry Floor	Refrig-erator Door	Dog Bowl
AMP AMC ATM CEF CAZ GEN STR TOB KAN CIP NAL TET CHL SXT	1									4
AMP ATM CEF CAZ GEN STR TOB KAN CIP NAL TET CHL SXT	19		5							
AMP ATM CEF CAZ STR CIP NAL TET CHL		6	4					2	1	4
None	8		6	4	3	3	2			
STR KAN NAL TET				3						
AMP ATM CEF CAZ				4						
AMP ATM CEF				2						
AMP ATM CEF CAZ CTX GEN STR KAN TET CHL SXT					1					
AMP ATM CEF CAZ CTX STR TOB TET CHL STX					5					
AMP CEF CTX STR KAN TET CHL SXT					1					
CEF KAN CIP NAL TET CHL SXT					11					
KAN CIP NAL TET CHL SXT						6				
AMP GEN TOB CIP NAL						1				
AMP STR TET SXT						2				
STR CIP TET SXT						1				
TET						1				
AMP ATM CEF STR KAN TET							1			
AMP STR KAN TET SXT							3			
AMP SRT KAN TET							3			
AMP STR NAL SXT							1			
TET SXT							1			
AMP AMC ATM CEF GEN SRT TOB KAN AMK TET CIP NAL CHL SXT										1
AMP ATM CEF GEN STR TOB KAN CIP NAL CHL SXT										1
AMP AMC ATM CEF CAZ STR CIP NAL TET CHL								2	1	

[a]Abbreviations: Ampicillin (AMP), amoxicillin-clavulanic acid (AMC), aztreonam (ATM), cephalothin (CEF), ceftazidime (CAZ), cefotaxime (CTX), gentamicin (GEN), amikacin (AMK), streptomycin (STR), tobramycin (TOB), kanamycin (KAN), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), chloramphenicol (CHL), and trimethoprim-sulfamethoxazol (SXT).

1 µL was streaked on Chromocult tryptone bile X-glucuronide (TBX) agar and 100 µL were spread on the same culture media containing cefotaxime (2 µg/mL). Oral swabs from the dog and house environmental swabs were put on buffered peptone water (BPW). After one hour at room temperature, unsupplemented and cefotaxime-supplemented TBX agar plates were inoculated with 100 µL from each sample. The urine was employed directly with 1 µL streaked on TBX agar and 100 µL spread on TBX containing cefotaxime at the same concentration.

Plates were incubated overnight at 37°C. Five colonies with the typical appearance of *E. coli* were selected from each plate and all colonies presenting different morphologies were additionally picked. Standard biochemical methods were used for the confirmation of *E. coli* isolates (Berge et al., 2006). The present procedure was adapted from standard protocols (Costa et al., 2008a; Simões, Poirel, Costa, & Nordmann, 2010) used in related studies as long as it is performed for getting the most reliable and accurate *E. coli* detection.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was performed using disk diffusion assay, following Clinical and Laboratory Standards Institute (CLSI, 2007) guidelines. Briefly, fresh bacterial colonies were inoculated on BPW suspension to a turbidity equivalent to 0.5 McFarland standard. With a sterile cotton swab the culture was swabbed on 150 mm depth Mueller-Hinton agar plates and standard discs (Oxoid antimicrobial susceptibility test discs) were applied using a disk dispenser. A total of 19 antimicrobial agents were tested: ampicillin, amoxicillin-clavulanic acid, aztreonam, cephalothin, ceftazidime, cefotaxime, cefoxitin, imipenem, gentamicin, amikacin, streptomycin, tobramycin, kanamycin, ciprofloxacin, nalidixic acid, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazol, and nitrofurantoin.

The antimicrobials were selected because they, or their related antimicrobials, had been regularly used in both human and veterinary medicine and to provide diversity in representation of different antimicrobial classes (Elseviers, Ferech, Vander Stichele, Goossens, & ESAC Project Group, 2007; Goossens et al., 2005). The reference strain *E. coli* American Type Culture Collection 25922 was included as a control. After 10 hours of incubation at 37°C, the diameters of the inhibition zones were measured using a caliper rounded up to the next millimeter and recorded. The interpretation of the inhibition zone length was made according to CLSI recommendations and breakpoints for Enterobacteriaceae.

According to several related studies (Costa et al., 2008a; Simões et al., 2010) quantitative analysis of antimicrobial resistance data was performed through a few basic descriptive statistic measures.

Polymerase Chain Reaction Amplification of Antimicrobial-Resistant Genes

Characterization of antimicrobial-resistant genes was performed in all strains displaying different antimicrobial resistance phenotypic patterns and strains with similar resistance patterns but isolated from different sources (humans, pets, or household environment). Bacteria were subcultured from glycerol stored cultures on tryptone soy agar medium overnight and DNA was extracted. Genomic DNA was extracted *in situ* by treatment with lysozyme (1 mg/mL) and proteinase K (0.5 mg/mL).

Genes for testing were selected taking into consideration the groups of antimicrobial drugs represented in the resistance phenotypes. Primers sequences and predicted sizes employed for polymerase chain reaction (PCR) amplification of the different antimicrobial-resistant genes are presented in Table 1 (Costa et al., 2008a; Costa et al., 2008b; Eckert et al., 2004; Mendonça, Leitão, Manageiro, Ferreira, & Caniça, 2007; Sáenz et al., 2004; Sidjabat et al., 2006; Srinivasan et al., 2007). In β-lactam resistant phenotypes, the presence of *amp*C, bla_{TEM}, bla_{OXA}, bla_{SHV}, bla_{CTX-M}, and $bla_{CTX-M-15}$ genes was studied. For aminoglycosides-resistant isolates, genes *str*A, *str*B, and *aad*A were investigated whereas phenotypes resistant to quinolones were explored for *gyr*A and *par*C genes. Tetracycline-resistant phenotypes were examined for *tet*A and *tet*B genes while the *cml*, *flo*, and *cat*A genes were explored in isolates showing resistance to phenicols. Genes *sul*1 and *sul*2 were scrutinized in isolates that showed resistance to trimethoprim-sulfamethoxazole.

Antimicrobial-resistant gene primers were obtained from previous studies (Costa et al., 2008a; Costa et al., 2008b; Eckert et al., 2004; Mendonça et al., 2007; Sáenz et al., 2004; Sidjabat et al., 2006; Srinivasan et al., 2007), as well as the amplification protocol. Primer sets were synthesized by Stab Vida. Amplification was performed in a DNA thermal cycler with 46-well PCR plates of 0.5 mL. The Taq polymerase kit used was from Fermentas Life Sciences. The reaction mixture consisted of 30 µL of sterile water; 5 µL of 10X Taq Buffer (100 mM Tris–HCl [pH 8.8 at 25°C], 500 mM KCl, 0.8% Nonidet P40); 1.5 µL of 25 mM $MgCl_2$; 1 µL of deoxyribonucleoside triphosphates (2 mM each dATP, dCTP, dTTP, and dGTP); 0.5 µL of each primer (stock concentration, 40 µM); 10 µL of template; and 0.2 µL (5 U/µl) of TaqDNA polymerase. Preincubation was at 94°C for five minutes.

Thirty PCR cycles were run under the following conditions: denaturation at 94°C for 30 seconds, primer annealing at optimum temperature for 30 seconds, and DNA extension at 72°C for 30 seconds in each cycle. After the last cycle, PCR tubes were incubated for seven minutes at 72°C and held at 4°C. The annealing temperature was optimized for all primer sets. *Salmonella typhi* G8518 CDC (ACCuST) and *Salmonella typhimurium* DT104 and DT193 were used as positive controls in all PCR reactions. With the exception of template DNA, sterile distilled water was used as the reagent control in the reaction mixture. The reaction mixture (20 µL) was analyzed by standard submarine gel electrophoresis (1.5% agarose; 5 V), and visualized by staining with ethidium bromide (0.5 mg/mL in the running buffer).

Results

The questionnaire submitted to the owners about themselves as well as the relationship with their pets, the respective human and animal medical records, in addition to some social interaction patterns and routines resulted in some useful information. Both husband and wife were 62 years old. The woman always worked at home as a seamstress and the man had an administrative job at the central mail services. The nine-year-old dog in question had a chronic, poorly controlled, allergic skin disease with recurrent pruritus and pyoderma. To control the secondary skin infections, several antimicrobial treatments had been prescribed: amoxicillin and clavulanic acid, cephalosporin, cefovecin, enrofloxacin, and ciprofloxacin.

The medical record of the man contained also an important detail: when hospitalized after a car accident (four years ago) he contracted a urinary tract infection treated according to the hospital protocol. The hospitalization lasted around two months, nearly the time he was under antimicrobial treatment. No relevant information was detected in the medical records of the woman, the child, or their exclusively indoor 12-year-old cat. The family lived in a small central apartment with apparently a medium economic level and good hygiene habits. The dog was dominant and an active element of the family with free access to all the divisions and items within the house. It was walked throughout the city center twice a day with a leash. Both pets, living with the owners since birth, were fed with specific canned dry food and drank water from the public system while their owners drank bottled water only.

A total of 124 *E. coli* isolates were recovered from the 17 samples collected from pets, hu-

TABLE **3**

Genes of Antimicrobial Resistance Found in *E. coli* Isolates From Pets, Human Cohabitants, and Household Environment

Resistance Genes	Pets				Human Cohabitants			Household Environment		
	Dog Feces	Dog Urine	Dog Mouth	Cat Feces	Male Feces	Female Feces	Child Feces	Laundry Floor	Refrig-erator Door	Dog Bowl
ampC	+	+	+	+	+	+	+	+	+	+
bla_{TEM}		+			+	+	+			
bla_{OXA}	+	+	+				+	+	+	+
bla_{SHV}	+	+	+	+				+	+	+
bla_{CTX-M}					+					
$bla_{CTX-M-15}$					+					
strA					+	+	+			
strB					+	+	+			
aadA	+	+	+	+	+	+	+	+	+	+
gyrA	+	+	+	+	+	+	+	+	+	+
parC	+	+	+	+	+	+	+	+	+	+
tetA					+	+	+			
tetB	+	+	+	+	+			+	+	+
cml					+	+				
flo	+		+		+					+
catA	+	+	+					+		+
sul1	+	+			+	+	+			+
sul2	+	+			+	+	+			+

mans, and household environment. The number of isolates, their location, and resistance profiles are presented in Table 2. No cultivable *E. coli* was obtained from light switches, the dog's water bowl, or owners' hands.

Antimicrobial susceptibility testing displayed 24 different phenotypic patterns with a remarkable representation of multiresistant ones. Fifty-seven isolates (46%) displayed simultaneous resistance to at least nine different antimicrobials. Six *E. coli* isolates obtained from the dog's food bowl and feces were resistant to 14 out of the 19 antimicrobials tested. A considerable proportion of the *E. coli* isolates displayed resistance to tetracycline (75%), ampicillin (64%), streptomycin and chloramphenicol (60%), nalidixic acid (59%), cephalothin (58%), trimethoprim-sulfamethoxazole (53%), kanamycin (51%), ciprofloxacin (48%), and aztreonam (47%). The percentage of resistance to the other antimicrobial agents was below 28% and no resistance against cefoxitin, imipenem, or nitrofurantoin was detected.

It is noteworthy that the same resistance phenotype that displayed simultaneous resistance against nine antimicrobials was found in samples collected from the dog (urine and mouth swab) and in household environmental samples, namely from the laundry floor, the refrigerator door, and the dog's food bowl (Table 2). The resistance pattern of some other strains isolated from the dog (feces and urine) also matched some of those found in the dog's mouth, food bowl, laundry floor, and refrigerator door.

The results of the antimicrobial-resistant gene detection using PCR are presented in Table 3. The pool of antimicrobial resistance genes encountered in isolates obtained from the dog's feces, urine, mouth, and food bowl and the owners' feces comprised resistance to all of the tested antimicrobial groups. Phenicol was the only antimicrobial group to which no resistant genes were found in isolates from the grandchild's feces. Of the resistant genes tested

for, phenicol and trimethoprim-sulfamethoxazole resistance was absent in isolates from the cat and from the refrigerator door, and trimethoprim-sulfamethoxazole resistance was absent in isolates from the laundry floor.

From a total of 18 genes tested, *E. coli* isolated from the man's feces carried a total of 15 antimicrobial-resistant genes (Table 3). Isolates from feces, urine, mouth, and food bowl of the dog held 11, 11, 9, and 11 resistant genes, respectively. Strains isolated from feces of the woman and the grandchild carried 11 resistant genes. Analysis of the isolates from the laundry floor, the refrigerator door, and from the cat's feces resulted in 8, 7, and 6 resistant genes, respectively. The woman, the grandchild, and the cat, although never subjected to antimicrobial treatments, demonstrated to have multiresistant isolates with some common resistance patterns (Table 3).

Discussion

The aim of our study was to obtain a holistic picture of the in-house *E. coli* antimicrobial resistance profiles, accounting for the contribution of the different pet and human cohabitants as well as the household surfaces and objects.

Interesting results were achieved, namely the high level of antimicrobial resistance found in the majority of the isolates attained (Table 2), which is remarkable when compared with similar studies undertaken previously (Carattoli et al., 2005; Costa et al., 2008a; Machado et al., 2007; Mendonça et al., 2007; Moreno, Bello, Guggiana, Dominguez, & Gonzalez, 2008; Normand, Gibson, Reid, Carmichael, & Taylor, 2000). The finding of a higher prevalence of antimicrobial resistance among *E. coli* strains isolated from the dog and the male owner was somewhat expected considering their history of antimicrobial treatments, including the man's hospitalization, which is known to increase the risk for acquiring, temporary or permanently, multiresistant strains (Dancer, 2004; Mendonça et al., 2007).

It is also noteworthy that strains isolated from the household environment, besides being resistant to at least nine of the tested antimicrobials, were found to have similar resistance profiles when compared to those from the home inhabitants, particularly those from the dog (Tables 2 and 3). Others have already found that the virulent human pathogen *E. coli* serotype O157, of whom cattle are the primary reservoir, remain viable in soil fecal excretion greater than four months (Jones, 1999) or in wood samples from farmyard material (Williams, Avery, Killham, & Jones, 2005).

To our knowledge, this was the first time that *E. coli* from household environment samples was analyzed and its antimicrobial-resistant determinants compared with those isolated from the household inhabitants. These findings raise questions regarding the potential contribution of shared household surfaces in antimicrobial resistance transfer between animal and human cohabitants. Finally it was established that a pet can orally transport *E. coli* strains with the same antimicrobial resistance profile of their fecal and urinary strains, which could be explained by some frequent behavior of dogs such as rolling on feces, grooming, and perigenital licking. The presence of those resistant strains in the dog's mouth is likely to have played a key role in their spread.

Commensal flora of the grandchild, the woman, and the cat have never been directly exposed to antimicrobial drugs; however, several multiresistant *E. coli* were also recovered from their stool samples and, more importantly, those strains shared most of the resistant genes found in those recovered from the dog and the man (Table 3). This finding is not so surprising for the woman since she is the man's sex partner, which is known to have a risk of acquiring their commensal *E. coli* (Johnson et al., 2008) but results in an interesting picture if we hypothesize that the cat, who never lived outside the home, acquired antimicrobial resistances to ampicillin, aztreonam, cephalothin, and ceftazidime, all frequently expressed in isolates of the dog, the man, and the household, through the normal cohabitation contacts. The same could be speculated concerning the child's antimicrobial resistance patterns.

Conclusion

Although resistance patterns are not static the genotypic and phenotypic correspondences demonstrated in this applied study could suggest interspecies transmission. Furthermore, the finding that almost all of these resistant genes were also present among strains isolated from the household environment could be indicative of an in-home and through-home transmission.

While concurrent colonization with multiresistant *E. coli* has been identified in humans and animals (Guardabassi et al., 2004a; Johnson et al., 2001; Johnson et al., 2008), our study provides further information that supports the potential contribution of the household environment as a passive source of multiresistant *E. coli* that could be acquired by touching contaminated surfaces or objects. Thus, those strains could be repeatedly transmitted between humans and animals within the household aggregate. Further studies are needed to clarify how these strains were able to survive on physical surfaces (outside their natural environment), as this ability is one critical factor for indirect transmission to a new host or reinoculation on the original host.

Because resistance is becoming increasingly widespread without plausible relationships with the use of antimicrobials, it is necessary to consider other strategies to prevent the emergence of antimicrobial-resistant microorganisms. The phenotypic and genotypic correspondences found in our study could suggest interspecies transmission and support previous concerns that pets could become household reservoirs of multiresistant *E. coli* for subsequent infection (or reinfection) of susceptible household members. Johnson and co-authors (2001) corroborated these findings by confirming that canine feces can be regarded as a reservoir for virulent human clones of extraintestinal pathogenic *E. coli*. Frequent within-household sharing of *E. coli* strain was demonstrated among pets, humans, sex partners and non-sex partners (Johnson et al., 2008). This paradigm of in-home and through-home *E. coli* spreading patterns and antimicrobial-resistant genes transfer could influence the design of preventive measures against the diffusion of pathogenic organisms or antimicrobial-resistant genes throughout the population.

Corresponding Author: Liliana Raquel Leite Martins, Institute for the Biomedical Sciences Abel Salazar, UP VET Small Animal Veterinary Clinic, Porto University, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. E-mail: lmartins@icbas.up.pt.

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2.2. HOUSEHOLD ANTIMICROBIAL RESISTANCE SHARE AND SPREAD

2.2.1. Paper IV

SPREAD OF MULTIDRUG-RESISTANT *ESCHERICHIA COLI* THROUGH DOMESTIC AGGREGATES (HUMANS, PETS AND HOUSEHOLD ENVIRONMENT)

Leite-Martins, L., Meireles, D., Beça, N., Bessa, L.J., de Matos, A.J.F., da Costa, P.M.

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Spread of multidrug-resistant Escherichia *coli* within Domestic Aggregates (humans, pets and household environment)

Liliana Leite-Martins[1,2*], Diana Meireles[2], Nuno Beça[2], Lucinda J. Bessa[2,3], Augusto J. F. de Matos[1,4] and Paulo Martins da Costa[2,3]

[1] Department of Veterinary Clinics – UPVET, Abel Salazar Institute for the Biomedical Sciences (ICBAS), University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal;

[2] Laboratory of Microbiology and Food Technology, ICBAS, University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal;

[3] Interdisciplinary Center for Marine and Environmental Research (CIIMAR), University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal;

[4] Animal Science and Study Centre / Food and Agrarian Sciences and Technologies Institute CECA/ICETA), University of Porto, Rua D. Manuel II, Apartado 55142, 4051-401 Porto, Portugal.

Abstract

Advances in veterinary medicine are largely associated with recourse to antimicrobial therapies, paving the way to the emergence of resistant bacteria, potentially able to spread globally. The aim of this study was to elucidate the manner in which shared environments between pets receiving antimicrobial treatments and their owners can potentiate the spread of antimicrobial-resistant *Escherichia coli*. Three domestic aggregates (DA), including pets, owners and household environment were studied. Each core pet had history of previous antimicrobial therapies. Overall, 231 E. *coli* isolates were recovered and analyzed by antimicrobial susceptibility testing. Subsequently, some isolates were eligible to carry out ERIC-PCR and PFGE analyses, in order to evaluate their genetic relatedness. The three core dogs presented multidrug-resistant *E. coli* clones disseminated over various body sites. In DA A and B, clonal disseminations among animals, owners and household surfaces were observed. In conclusion, results highlighted the spread of multidrug-resistant *E. coli* within household.

Key words: antimicrobial resistance, *Escherichia coli* spread, pets, humans, household surfaces.

1. Introduction

Antimicrobial resistance (AMR) is a major public health problem worldwide and will probably be the main global concern of the next decade (Carlet, *et al*., 2012). The phenomenon of AMR is a complex problem involving several bacterial species, resistance mechanisms, transfer mechanisms and reservoirs (Guardabassi *et al*., 2004). Although the major consequences of AMR are more noticeable in the clinical setting, emergence and dissemination of resistance is mainly to happen in the environmental microbiota, where studies aimed to fully understand the cycle of acquisition of resistance by human pathogens are needed (Martínez, 2012).

The notorious improvement of companion animals' medical assistance was accompanied by the appearance of veterinary hospitals and the adoption of clinical procedures similar to the ones used in human medicine. Hospitalized pets under antibiotic treatment have provided a scenario that strongly favors the occurrence and dissemination of AMR (Hall *et al*., 2013) similar to what happens in human clinical settings. When these animals are discharged and go home, due to the close contact and contempt in hygiene practices between owners and their pets, they can easily transfer antimicrobial-resistant strains (Guardabassi *et al*., 2004; Lloyd, 2007; Murphy *et al*., 2009) directly (via contact with skin, saliva or feces) or indirectly (via the household environment) to their animal or human cohabitants (Martins *et al*., 2013).

Escherichia coli can be used to track the evolution of antibiotic resistance in different ecosystems not only due to its important role as acceptor and donor of transmissible drug resistance genes, from and to pathogenic bacteria (van den Bogaard and Stobberingh, 2000; Sáenz *et al*., 2004), but also because it is commonly found in the intestinal tract of humans and animals and widely spread in fecal contaminated water, soil and food (Costa *et al*., 2008; Murphy *et al*., 2009; da Costa *et al*., 2013).

The above concerns led us to pursue three hypotheses throughout the present work: i) the possibility of *E. coli* from dog feces to colonize other body sites of the animal; ii) the ability of that same *E. coli* disseminate to household surfaces and objects and iii) the occurrence of intra-species and inter-species *E. coli* transmission within the same domestic aggregate.

Accordingly, we conducted a cross-sectional point prevalence survey of *E. coli* colonization patterns in three domestic aggregates. Cefotaxime supplemented media was utilized to facilitate the recovery of low-frequency clones and enterobacterial repetitive

intergenic consensus-polymerase chain reaction (ERIC-PCR) and pulsed-field gel electrophoresis (PFGE) were the tools used to assess *E. coli* genetic diversity from humans, pets and household surfaces.

2. Materials and Methods

2.1. Study design and compliance

Domestic Aggregates (DA) integrating this study emerged from the universe of clients of the Veterinary Hospital of the University of Porto (UPVet). Eligibility criteria for this study branch required that the core pet (the animal visiting the hospital), from the applicant domestic aggregate (including owners and other pets), had been submitted to at least one antimicrobial treatment over the previous 6 months. The owners were asked to sign in a term of acceptance; to fill a questionnaire about intrinsic and environmental variables of each one of the DA elements, including human and veterinary medical information regarding antibiotic exposure; to bring their own stool samples and to allow the collection of swabs from their hands; fecal, urinary and oral secretions samples and skin and fur swabs from their pets as well as swabs from commonly touched household objects and surfaces (light switches, door knobs, TV remote control, mobile phones, banister, refrigerator door handle, kitchen floor, pets beds, leash, food and water recipients). Approval was obtained from the Ethics Committee of the Abel Salazar Institute for the Biomedical Sciences, University of Porto.

2.2. *Escherichia coli* isolation

Fecal samples were immediately diluted 1:10 in saline buffer and stored at room temperature for 30 min. From the initial suspension, an aliquot of 5 µl was streaked on Tryptone Bile X-glucuronide agar (TBX; Biokar Diagnostics, Allonne, Beauvais, France) and 100 µl were spread on the same culture media containing 2 µg/ml of cefotaxime (Sigma-Aldrich, St. Louis, MO, USA). The urine was applied directly by streaking 5 µl on TBX agar and 100 µl on TBX containing cefotaxime. The swabs were immersed on Buffered Peptone Water (BPW; Oxoid, Basingstoke, Hampshire, England) for 30 min at room temperature and, subsequently, 100 µl were spread on non-supplemented and cefotaxime-supplemented TBX agar plates.

Plates were incubated overnight at 37°C. A maximum of five colonies with typical appearance of *E. coli* were selected from each non-supplemented TBX agar plate and all colonies presenting different morphologies were additionally picked from the cefotaxime supplemented TBX agar plates. Standard biochemical methods were used for the confirmation of *E. coli* isolates (Berge *et al*., 2006). The described procedure was adapted from standard protocols (Costa *et al*., 2008; Martins *et al.*, 2013) used in related studies aiming to achieve the most reliable and accurate *E. coli* detection.

2.3. Antimicrobial susceptibility characterization

Disk diffusion assay, following CLSI guidelines (CLSI, 2012), was performed to assess the antimicrobial susceptibility of each isolate. Selected antimicrobial drugs included those regularly used in both human and veterinary medicine and were representative of different antimicrobial classes. A total of 19 antimicrobial agents (Oxoid) were tested: ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg), aztreonam (ATM, 30 µg), cephalothin (CEF, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), cefoxitin (FOX, 30 µg), imipenem (IPM, 10 µg), gentamicin (GEN, 10 µg), amikacin (AMK, 30 µg), streptomycin (STR, 10 µg), tobramycin (TOB, 10 µg), kanamycin (KAN, 30 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NAL, 30 µg), tetracycline (TET, 30 µg), chloramphenicol (CHL, 30 µg), trimethoprim-sulfamethoxazol (SXT, 25 µg) and nitrofurantoin (NIT, 300 µg).

2.4. DNA extraction and *E. coli* phylogenetic group determination

Multidrug-resistant bacteria were considered according to previous reported definition (Magiorakos *et al*., 2011). Criteria designed for selecting the group of isolates, from each DA, eligible for genetic analysis were: i) multidrug-resistant *E. coli* with different antimicrobial resistance patterns and ii) multidrug-resistant strains that had similar antimicrobial resistance phenotypes but isolated from different sources.

The isolates were cultured in Müeller-Hinton agar (MH; Biokar Diagnostics) at 37ºC and harvested at late exponential phase to perform DNA extraction by using the InstaGene Matrix ® (Bio-Rad Laboratories, California, USA) as described by the manufacturer. A simple and rapid phylogenetic grouping technique based in a triplex PCR was applied, as already described (Clermont *et al.*, 2000).

2.5. ERIC-PCR fingerprinting

A 25-µl ERIC-PCR reaction was carried out using the primers ERIC-1R (5'-ATG TAA GCT CCT GGG GAT TCA C) and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G) as previously described (Versalovic *et al*., 1991; Meacham *et al*., 2003). The PCR amplifications were performed in a DNA thermal cycler MyCycler® (Bio-Rad Laboratories), with an initial incubation at 94ºC for 3 min, followed by 30 cycles consisting of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 3 min. A final extension at 72ºC for 5 min was programmed to complete the amplification (Leung *et al*., 2004).

The ERIC-PCR patterns of each isolate was visualized after electrophoresis for 45 min at 150 V using a 1.5% agarose gel containing 1x TBE buffer (National Diagnostics, Atlanta, GA, USA) and 0.5 µg/ml ethidium bromide. Gels were photographed using a Molecular Imager Gel Doc XR® (Bio-Rad Laboratories).

2.6. PFGE fingerprinting

All eligible isolates from DA A and DA C were used for both ERIC-PCR and PFGE analyses; however, given the high number of isolates selected from DA B and the respective ERIC-PCR results, some isolates exhibiting lower ERIC fingerprinting similarity (< 85%) and coming from different sources or high similarity but originated from the same source were excluded from PFGE analysis. PFGE fingerprints, obtained using XbaI-digested total DNA, were interpreted by using previous criteria (Johnson *et al*., 2008; Stenske *et al*., 2009). According to Tenover *et al*. (1995), isolates can be considered clonally related if their fingerprinting profiles do not differ in more than two or three bands.

Pulsed-field gel electrophoresis was performed as previously described (Barret *et al*., 1994; Ejrnaes *et al*., 2006), with minor modifications. In brief, a single pure colony of each isolate was inoculated in BPW and incubated overnight, at 37ºC. Then, OD_{600} was adjusted to 1.0 and bacterial suspensions were pelleted and washed with a suspension buffer (10mM Tris Buffer, pH7.5; 20 mM NaCl; 50 mM EDTA, pH8.0), and mixed with an equal volume of melted LMP agarose at 2% (SeaPlaque Agarose *low melting temperature*). The mixture was dispensed into plug molds. After solidification, agarose plugs were transferred to the lysis buffer (10 mM Tris Buffer, pH7.5; 50 mM NaCl; Na deoxychotalo 0.2%, Na laurylsarcosine 1% ,1 mg/ml lisozyme) and incubated at 37ºC for 2 h. Lysis buffer was removed, and plugs were washed with sterile distilled water for 5 min, followed by overnight incubation at 50ºC in proteinase K buffer (100mM EDTA pH8,

Na deoxycholato 0.2%, Na laurylsarcosine 1%, 1 mg/ml proteinase K). After lysis, plugs were washed 45 min in a PMSF buffer (20 mM Tris Buffer pH7.5, 50 mM EDTA, 0.7 mM PMSF) and four times in the same buffer but without PMSF. After a 30-min adaptation in 100 µl of restriction buffer (Buffer Tango 1X; Thermo Fisher Scientific, Waltham, MA, USA), plugs were transferred to the fresh mixture containing the restriction enzyme XbaI (10 U/µl; Thermo Fisher Scientific) at 40 U/100µl of plug, and incubated overnight at 37ºC. Then plugs were briefly soaked in standard Tris-borate-EDTA (TBE) 0.5x buffer, loaded into appropriate wells of the gel and sealed with melted 2% LMP agarose. Restriction fragments were separated by electrophoresis through 1% pulsed field agarose (Bio-Rad) in 2.5 l of standard TBE 0.5x buffer refrigerated at 14ºC, in a CHEF DR_III apparatus (Bio-Rad). Gels were run with a voltage of 6 V/cm and a linearly ramped pulse time of 4 to 36 s for a day. After electrophoresis, gels were stained with ethidium bromide (1 µg/ml) for 30 min, destained with distilled water for 15 min and photographed.

2.7. Data analysis

The assortment of all phenotypically characterized isolates, from each DA, was examined for the number of antimicrobial resistance determinants as well as for repetitive resistance patterns. For each DA, a collection with all multidrug-resistant strains with different antimicrobial patterns plus all multidrug-resistant strains with similar patterns but originated from different sources was created. The compilation of these isolates was used to build up the genetic component of the present study.

Similarities in ERIC-PCR and PFGE patterns were compared by means of the Dice coefficient using the Fingerprinting DST Molecular Analist Software (Bio-Rad Laboratories). Dendrograms were constructed by the unweighted pair group method using averages (UPGMA), and an optimization of 1% and position tolerance of 2.0% was applied. Strains were defined as representing the same strain (being indistinguishable or clonal) if they possessed ≥ 94% similarity in the PFGE profile (Johnson *et al*., 2008; Stenske *et al*., 2009) or defined as having a clonal relationship if they possessed ≥ 85% similarity (Ejrnaes *et al*., 2006).

3. Results

3.1. Domestic aggregate description

Two DA (A and B) allowed complete sampling (pets, owners and home environment) whereas DA C just allowed the core dog sampling.

DA A was composed of a core dog, a 14-year old spayed female Cocker Spaniel (Dog A1) that was chronically ill and had been treated repeatedly with multiple antimicrobial agents (Table 1). DA A included two more dogs, a 5-year old female Cocker Spaniel (Dog A2) and a 2-year old male Boxer (Dog A3), both healthy that had only visited the veterinary services for regular prophylaxis; they had never been ill and never took any antimicrobial drug. Their owners, a middle-age couple (Gentleman A and Lady A), were both healthy without recent antimicrobial treatments. The family lived in a peripheral urban villa with a garden, where dogs used to play (dog walking in the street rarely happened). All dogs were active elements of the family with free access to all the rooms and items within the house. The three pets lived with the owners since birth and were fed only with canned dry food. Dogs A2 and A3 used to have coprophagic habits when puppies.

The core dog from DA B (Dog B) was a 9-year old entire crossbreed female suffering from chronic, poorly controlled, allergic skin disease which required multiple courses of antimicrobial therapy (Table 1). Four years before, the dog owner (Gentleman B) had been hospitalized for two months, after a car accident and, in the hospital, contracted a urinary tract infection. No relevant information was detected in the medical history of the lady (Lady B), the 2-year old grandchild (Baby B) or their indoor 12-year old cohabitant female cat (Cat B). The family lived in an urban central small apartment. They were retired from public administration jobs. The dog was the dominant and the most active pet with free access to all rooms and items within the house and no restriction of interaction with the baby. It was walked throughout the city center twice a day, with a leash and had no coprophagic habits. Both pets lived with the owners since birth and were fed with canned dry food.

Domestic aggregate C allowed only the participation of the core dog. It was a 7-year old spayed female Saint Bernard suffering from a bladder tumor that demanded successive antimicrobial therapies as shown in Table 1. This DA was constituted by a middle-age couple and a 2-year old child; all three were quite healthy.

Table 1. Recent antimicrobial treatment histories of each Domestic Aggregate core pet

Animal	Antimicrobial drug	Protocol	Duration	End of treatment before sampling
Dog A1	Enrofloxacin	2.5mg/kg, PO, BID	6 weeks	3 weeks ago
	Amoxicillin-Clavulanic Acid	20mg/kg, PO, BID	2 weeks	3 months ago
Dog B	Ciprofloxacin	5mg/kg, PO, BID	4 weeks	2 weeks ago
	Enrofloxacin	2.5mg/kg, PO, BID	2 weeks	6 weeks ago
	Cefovecin	8mg/kg, SC, q14d	6 weeks	3 months ago
	Amoxicillin-Clavulanic Acid	20mg/kg, PO, BID	2 weeks	4.5 months ago
	Cephalexin	22mg/kg, PO, BID	2 weeks	6 months ago
Dog C	Enrofloxacin	2.5mg/kg, PO, BID	3 weeks	2 weeks ago
	Amoxicillin-Clavulanic Acid	22mg/kg, PO, BID	2 weeks	4.5 months ago

Legend: PO: *per os*; SC: subcutaneous; BID: each 12 h; SID: each 24 h; q14d: each 14 days.

3.2. *Escherichia coli* phenotypic and phylogenetic characterization

A total of 121 *E. coli* isolates were collected from DA A, the majority obtained from Gentleman A feces (n = 13), Dog A1 mouth (n = 11), Dog A2 feces (n = 11), Dog A1 hair (n = 10), Dog A3 feces (n = 10) and Lady A feces (n = 10). Antimicrobial susceptibility tests displayed 31 different phenotypic patterns, some of them being coincident in isolates from dogs, their owners and some environmental samples. The majority of this isolates were resistant to ampicillin, nalidixic acid, trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, chloramphenicol, cephalothin, tobramycin and cephoxitin. A remarkable proportion (89.2%) of multidrug-resistant strains was observed. No resistance was found towards aztreonam and nitrofurantoin.

From DA B, 101 isolates were recovered; the majority were isolated from Lady B feces (n = 17), Dog B feces (n = 15), Gentleman B feces (n = 15) and cat B feces (n = 13). Resistances to tetracycline, ampicillin, streptomycin and chloramphenicol were the most common amongst the tested isolates while all were sensitive to amikacin, nitrofurantoin,

cefoxitin and imipenem. Antimicrobial susceptibility tests displayed 20 different phenotypic patterns with an important representation of multidrug-resistant ones (75.0%) and there was an overlap among patterns of isolates from dog feces, mouth and environment samples.

In DA C, nine multidrug-resistant *E. coli* isolated from feces (n = 7), fur (n = 1) and oral secretions (n = 1) of Dog C displayed the same antimicrobial phenotype.

The compilation of the eligible isolates from DA A (n = 28), DA B (n = 17) and DA C (n = 3) for further genetic analyses, their respective antimicrobial resistance patterns and phylogenetic groups, are shown in Table 2.

3.3. *Escherichia coli* clonality

The genetic relatedness among *E. coli* isolates, from each DA, was examined by the use of ERIC-PCR and PFGE analysis. Pulsed-field gel electrophoresis is considered a "gold standard" technique for clonality studies (Goering, 2010), nevertheless, our ERIC-PCR results were a strong support for the overall outcome in view of the fact that both systems pointed toward the same directions (ERIC-PCR results are shown in Fig. S1, Fig. S2 and Fig. S3).

Considering a similarity cutoff of ≥ 94%, one single cluster (X1) was identified in DA A, as shown in Fig. 1. The fact that the strains appertaining to cluster X1 have similar antimicrobial resistance patterns and belong to phylogenetic group A strengthens the probable clonality of those strains. Considering that a similarity of 85% between PFGE patterns is enough to consider the isolates genetically related, all DA A studied strains (except Lady A feces, which was intentionally included, as control), could have a clonal relationship.

Regarding isolates from DA B, applying the criterion of ≥ 94% similarity to PFGE profiles (Fig. 2), three clusters of dissemination could be identified: Y1 (two isolates belonging to phylogenetic group B1 from Gentleman B feces); Y2 (Refrigerator door B 1, Dog B mouth 2, Kitchen floor B 1, Dog B mouth 1, Dog B feces 1 and Dog B food bowl 1) and Y3 (Gentleman B feces 1 and Dog B feces 2). Clone Y2 can be considered clonally related with Y3 taking into account the coefficient of similarity obtained (88.4%) and the phylogenetic group (all belong to phylogroup A). The remainder strains that had a similarity < 85%, either belong to different phylogroups or have more distinct antimicrobial resistance patterns, suggesting a probable diverse source.

Figure 3 displays the PFGE patterns of isolates from Dog C. A 100% similarity between the strains is perceived, suggesting a clonal (Z1) spread between feces, mouth and hair, supported by the same antimicrobial resistance pattern as well as the same phylogenetic group (phylogroup A).

4. Discussion

Firstly, it should be underlined that the problem of antimicrobial resistance is starting to catch the public attention. People are becoming aware about the consequences that the recurrent intake of antimicrobial drugs can have in the human and animal health; having families' agreement to participate in this study is a reflection of those concerns.

Secondly, the observations attained throughout the study of these three cases supported the hypotheses initially raised. Data obtained from DA A could be comparable to a multidrug-resistant *E. coli* outbreak, if transposed to an in-home scale. Results demonstrate that the same clonal strains, possibly emerged and disseminated from the feces of dog A1 (the element more often subjected to the selective pressure of antimicrobial treatments) to its own mouth, hair and skin and frequently touched objects for everybody in the house (dogs leashes, toys, food bowls and beds, the banister, the refrigerator door and the kitchen floor). Furthermore, the same clonal strain was found in the other two healthy dogs of the aggregate; a very likely explanation is a direct or indirect clonal intra-species transmission. Likewise, the same multidrug-resistant *E. coli* clone appeared in Gentleman A hands and feces, sustaining an inter-species dissemination. The findings obtained from DA B supported the outcomes from DA A. Again multidrug-resistant *E. coli* clones were found in different body sites of the dog (feces and mouth) as well as through some household surfaces (kitchen floor, refrigerator door and dog food bowl) and were closely related (> 85%) to another cluster of two isolates found in the dog and owner feces. Findings from Dog C confirmed the possibility of fecal *E. coli* clones to colonize other body sites of the same individual. Clonal spread was supported with 100% similarity, using both techniques (ERIC-PCR and PFGE), in feces, mouth and hair.

Table 2. Antimicrobial resistance patterns and phylogenetic groups of isolates studied through PFGE, in each domestic aggregate

DA	Isolate Source	Antimicrobial Resistance Pattern	PG
DA A	Dog A2 Leash	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A2 Food Bowl	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A2 Toy	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A2 Feces	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A2 Mouth	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A2 Hair	AMP, FOX, IPM, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Feces 2	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Banister A 2	AMP, FOX, CIP, GEN, TET, CTX, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Gentleman A Hands	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Kitchen Floor A	AMP, FOX, CIP, GEN, TET, CTX, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Gentleman A Feces 1	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Feces 1	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dogs bed A	AMP, FOX, CIP, GEN, TET, CTX, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Mouth	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Hair 1	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Skin	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Food Bowl 2	AMP, FOX, CIP, GEN, TET, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A3 Feces	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A3 Leash 1	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A3 Leash 2	AMP, FOX, CIP, GEN, TET, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A3 Food Bowl	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Banister A 1	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Refrigerator door handle A	AMP, FOX, CIP, GEN, TET, CTX, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A3 Mouth	AMP, FOX, IPM, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Hair 2	AMP, FOX, CIP, GEN, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Gentleman A Feces 2	AMP, FOX, CIP, GEN, CAZ, AMC, CEF, AMK, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog A1 Food Bowl 1	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Lady A Feces	AMP, STR	B2
DA B	Lady B Feces 3	CIP, TET, NAL, CHL, SXT, KAN	B1
	Cat B Feces 2	TET, STR, NAL, KAN	B1
	Gentleman B Feces 3	AMP, TET, CTX, ATM, CAZ, CEF, STR, CHL, STX, TOB	B1
	Gentleman B Feces 4	AMP, GEN, TET, CTX, ATM, CAZ, CEF, STR, CHL, TOB, SXT, KAN	B1
	Dog B Urine	AMP, CIP, TET, ATM, CAZ, CEF, STR, NAL, CHL	B1
	Refrigerator door B 1	AMP, CIP, TET, ATM, CEF, STR, NAL, CHL	A
	Dog B Mouth 2	AMP, CIP, TET, ATM, CAZ, CEF, STR, NAL, CHL	A
	Kitchen Floor B 1	AMP, CIP, TET, ATM, CAZ, CEF, STR, NAL, CHL	A
	Dog B Mouth 1	AMP, CIP, GEN, TET, ATM, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog B Feces 1	AMP, CIP, GEN, TET, ATM, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Dog B Food Bowl 1	AMP, CIP, GEN, TET, ATM, AMC, CEF, AMK, STR, NAL, CHL, TOB, SXT, KAN	A
	Gentleman B Feces 1	AMP, TET, CTX, ATM, CAZ, CEF, STR, CHL, SXT, KAN	A
	Dog B Feces 2	AMP, CIP, GEN, TET, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	A
	Lady B Feces 1	AMP, TET, STR, SXT	B1
	Lady B Feces 2	TET, STR, NAL, SXT	A
	Baby B Feces 1	AMP, STR, NAL, SXT	D
	Refrigerator door B 2	AMP, CIP, TET, ATM, CAZ, AMC, CEF, STR, NAL, CHL	D
DA C	Dog C Feces	AMP, AMC, CEF, CAZ, CTX, NAL, CIP, GEN, STR, CHL, KAN, ATM	D
	Dog C Mouth	AMP, AMC, CEF, CAZ, CTX, NAL, CIP, GEN, STR, CHL, KAN, ATM	D
	Dog C Hair	AMP, AMC, CEF, CAZ, CTX, NAL, CIP, GEN, STR, CHL, KAN, ATM	D

Legend: DA – domestic aggregate; PG – phylogenetic group

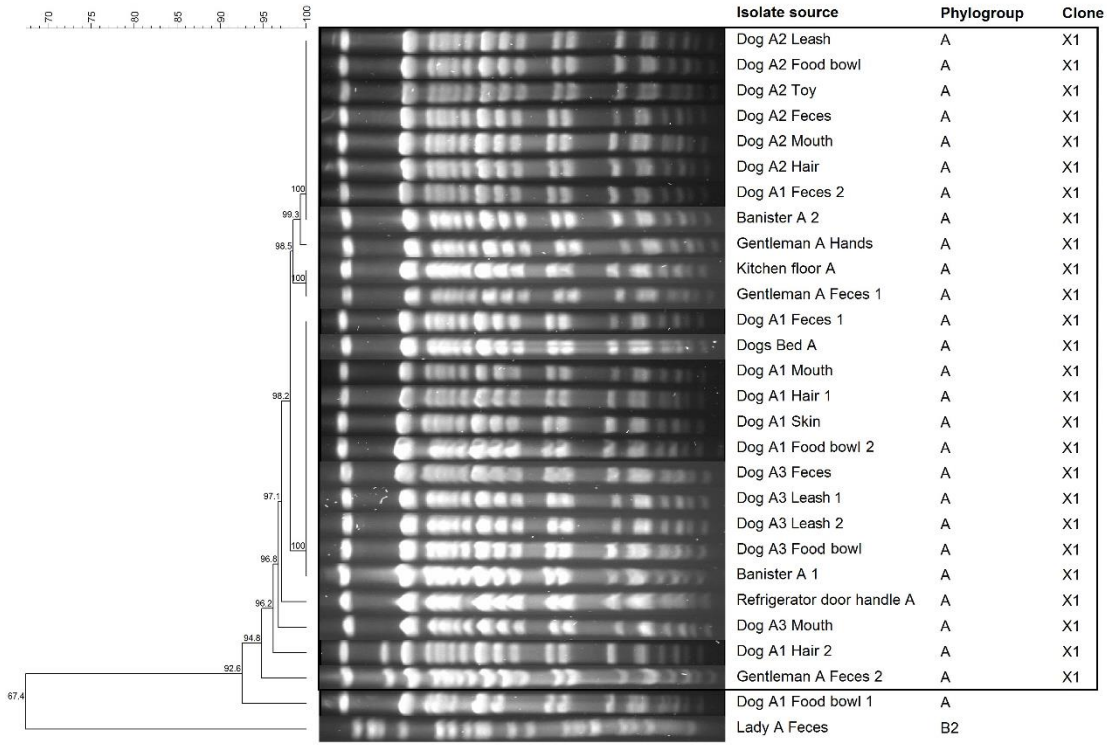

Fig. 1. Dendrogram showing genetic relatedness of selected isolates from DA A, determined by analysis of PFGE fingerprinting patterns using Dice similarity coefficient and UPGMA cluster method.

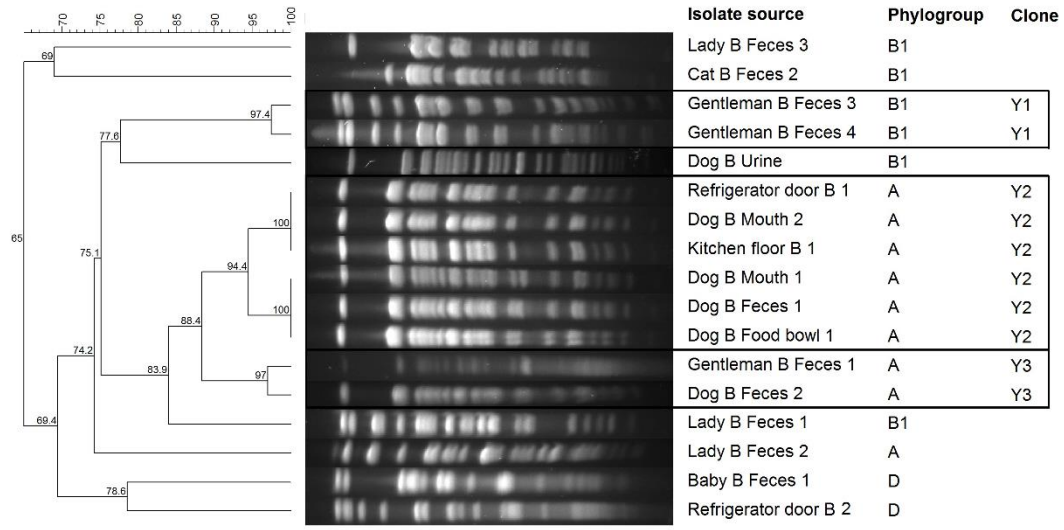

Fig. 2. Dendrogram showing genetic relatedness of selected isolates from DA B, determined by analysis of PFGE fingerprinting patterns using Dice similarity coefficient and UPGMA cluster method.

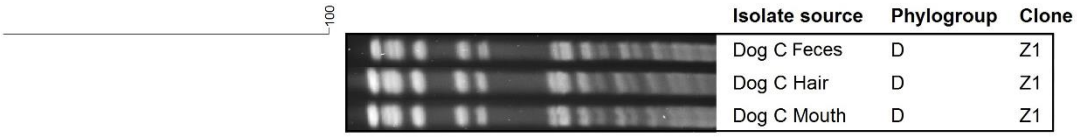

Fig. 3. Dendrogram showing genetic relatedness of selected isolates from DA C, determined by analysis of PFGE fingerprinting patterns using Dice similarity coefficient and UPGMA cluster method.

In this study, formulating conclusions about the direction of transmission was not attempted, although, it was demonstrated that dogs (in different body sites), owners and household surfaces can share *E. coli* isolates with similar antimicrobial resistance profiles and ERIC-PCR and PFGE patterns, suggesting that within-household transmission may occur, in either direction, mediated directly by feces, oral secretions, urine, skin, fur, owners' hands or, indirectly, by contaminated household surfaces and objects. Rolling and rubbing on fecal material, grooming and perigenital licking are frequent behaviors of pets that together with their intrinsic hygienic limitations could predispose to the previous findings. None of the three core dogs had coprophagic habits that would justify mouth colonization with fecal bacteria.

The clonal dissemination between animals and human cohabitants in DA A and DA B may be explained by the intimate relationship that exists between owners and their pets, leading them to frequently neglect basic hygiene rules that are seldom forgotten in interactions with other humans or animals that are not part of their aggregate.

Besides the strains isolated from individuals, we also investigated strains isolated from the household environment. Our results demonstrate that several in-home surfaces may serve as a source of multidrug-resistant *E. coli* that is able to survive and persist outside the natural hosts long enough to potentially contaminate new hosts, including incoming visitors. Others have already found that the virulent human pathogen *E. coli* serotype 0157, whose primary reservoir is cattle, remain viable in soil fecal excretion for more than 4 months (Jones, 1999) or in wood samples from farmyard material (Williams *et al*., 2005). Garfield *et al*. (2008) highlighted that the duration of *E. coli* survival in canine feces is very dependent on the water content and evaporative conditions (under low evaporative conditions, *E. coli* can survive longer). Although the simultaneous colonization with multidrug-resistant *E. coli* has already been identified in humans and animals (Johnson *et al*., 2001; Johnson *et al.,* 2008; Platell *et al*., 2011), our study provides further

information in support of the potential contribution of the household environment as a passive source of multidrug-resistant *E. coli*. In fact, such strains could be acquired by touching the contaminated surfaces or objects and be repeatedly transmitted between humans and animals within the household, building up the in-home and through-home transmission mode.

In addition, it is well known that resistance harbors a fitness cost and it has been proposed that a reduction in antibiotic use would benefit the susceptible bacteria over the resistant ones; however, compensatory evolution and genetic co-selection also play a role, complicating the all scenario (Andersson and Hughes, 2010). Indeed, co-selection of resistance to more than one antibiotic, due to the genetic linkage between resistance genes, may explain the rise of resistance to an antibiotic that is not currently in use (Andersson and Hughes, 20101).

Further studies are needed to support and corroborate these findings as well as to better explore and characterize the interconnections and factors that drive the within-household antimicrobial resistance diffusion. Antimicrobial resistance is triggering a public health challenge, thus, understanding who or which are the participants in the transmission chain of resistance will eventually help to deploy new intervention strategies. Such strategies should take into account the important interconnections between human and animal health in accordance with the Manhattan principles on “One World, One Health” (da Costa *et al*., 2013).

Antimicrobial resistance is an emerging global problem, not just in the clinical settings (Tan *et al.*, 2013) but also in the community. As such, assessing the risk factors for the dissemination of drug-resistant bacteria, or their corresponding genetic material, between pets and their owners within household is essential for the implementation of safe handling procedures of companion animals and prudent use of antimicrobial substances in human and veterinary practice.

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Supplementary material

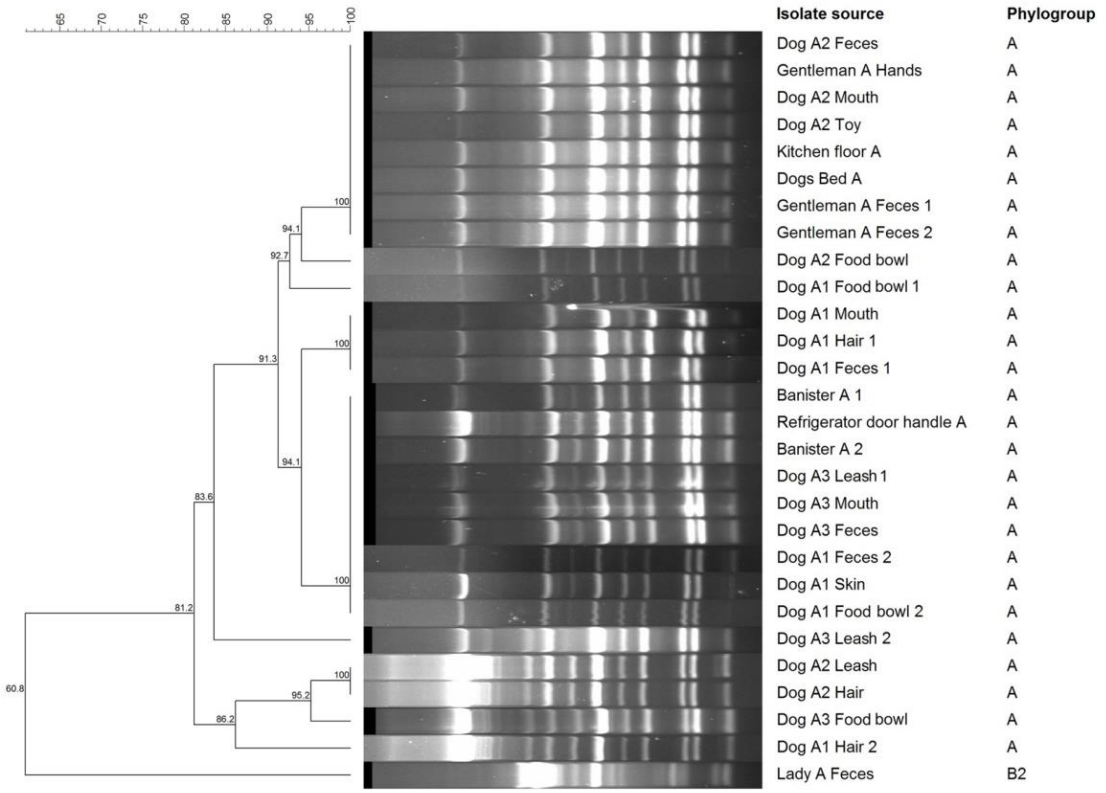

Fig S1. Dendrogram showing genetic relatedness of selected isolates from DA A, determined by analysis of ERIC-PCR fingerprint patterns using Dice similarity coefficient and UPGMA cluster method.

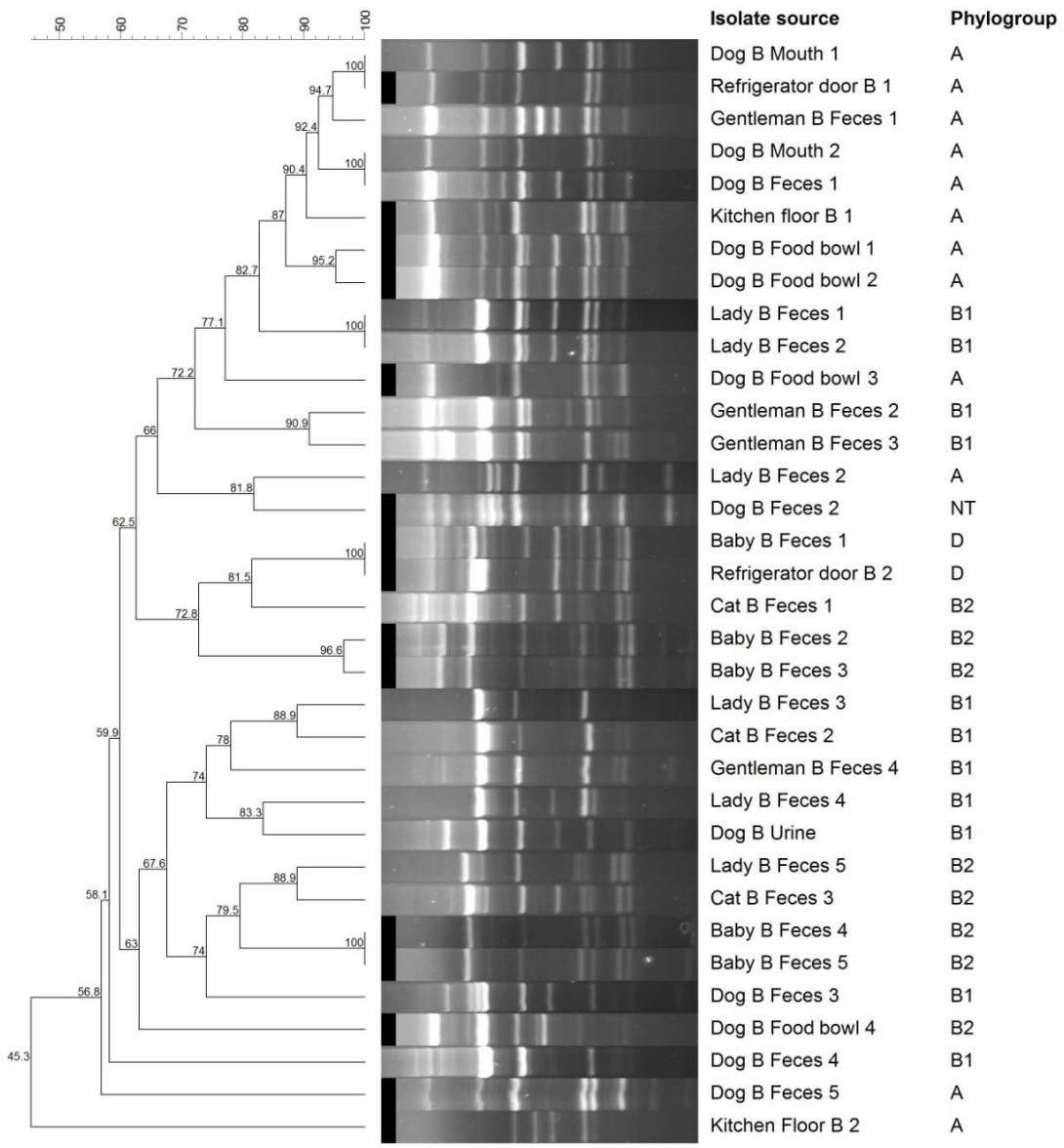

Fig S2. Dendrogram showing genetic relatedness of selected isolates from DA B, determined by analysis of ERIC-PCR fingerprint patterns using Dice similarity coefficient and UPGMA cluster method.

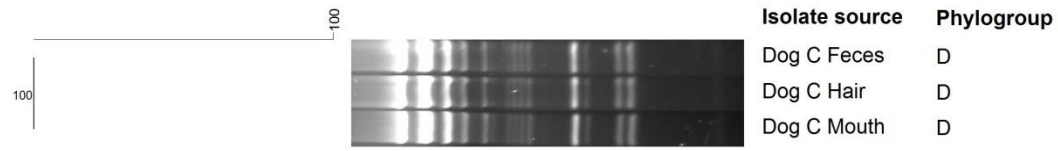

Fig S3. Dendrogram showing genetic relatedness of selected isolates from DA C, determined by analysis of ERIC-PCR fingerprint patterns using Dice similarity coefficient and UPGMA cluster method.

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2.2. HOUSEHOLD ANTIMICROBIAL RESISTANCE SHARE AND SPREAD

2.2.3. Paper V

SPREAD OF MULTIDRUG-RESISTANT *ENTEROCOCCUS FAECALIS* WITHIN THE HOUSEHOLD SETTING

Leite-Martins, L., Meireles, D., Bessa, L.J., Mendes, A., de Matos, A.J., Martins da Costa, P.

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Spread of Multidrug-Resistant Enterococcus faecalis Within the Household Setting

Liliana Leite-Martins,[1,2] Diana Meireles,[2] Lucinda J. Bessa,[3] Ângelo Mendes,[2] Augusto J. de Matos,[1] and Paulo Martins da Costa[2,3]

Advances in veterinary medicine have resulted in the survival of many animals with severe illness or infectious diseases. In addition, increased usage of antimicrobial agents for veterinary purposes has contributed to the worldwide problem of increasing antimicrobial resistance. The objective of this study was to contribute to better understand the potential and implications for the spread of antimicrobial-resistant enterococci between pets receiving antimicrobial treatments and their owners. Three household aggregates (HA A, B, and C) were selected for this study. Information was collected on individual and clinical parameters of both humans and animals that cohabit. For this study, samples of feces, oral secretions, skin and fur of pets, as well as owners' feces and hands and exposed household surfaces and objects were also collected. All enterococci isolates were analyzed for antimicrobial susceptibility. Based on the antimicrobial resistance patterns and origin of isolates, ERIC-PCR analysis was performed on selected isolates to evaluate phylogenetic relationships. In all three HA, Enterococcus faecalis clonal spread was detected between pets and the respective owners, confirming the in-home interanimal species dissemination. Additionally, fecal enterococci colonization of other body parts of the same animal and dissemination of those same enterococci to household surfaces and objects were also observed. Our results demonstrate that enterococcal clones were found in pets in multiple body sites, their human cohabitants, and shared domestic objects.

Introduction

It is well known that many antibiotic-resistant pathogens are able to cause a major clinical challenge in both human and veterinary contexts.[10,13] In particular, enterococci, ubiquitous in nature and a common commensal of the intestinal microbiota of people and animals, have emerged as one of the most prevalent nosocomial pathogens worldwide. This is due in part to inherent and acquired resistance to antimicrobials, putative virulence traits,[26] biofilm forming capability,[12] and ability to horizontally transfer antimicrobial resistance and virulence determinants to other bacteria.[7]

Advances in veterinary medicine and increased awareness of owners to the health and welfare of animals contributed to a higher usage of antimicrobial therapies, increasing the emergence of antimicrobial resistance among companion animals. Therefore, the longer and closest contact between pets and owners can facilitate the interspecies transfer of antimicrobial-resistant bacteria; this context certainly deserves more investigation and increased surveillance particularly because of the potential for bidirectional infection between animals and humans.[11,24] Dogs and cats are commonly colonized with antimicrobial-resistant enterococci and may act as reservoirs of antimicrobial resistance genes that can be transferred to people.[8,15,18,20]

These concerns led us to investigate the possibility of fecal enterococci present in dog feces to colonize other body parts of the animal and disseminate to household surfaces and objects, as well as the occurrence of intraspecies and interspecies enterococci transmission within the household aggregate (HA). We conducted a cross-sectional point prevalence survey of enterococci colonization patterns in three domestic aggregates. Enterococci diversity among humans, pets, and household surfaces was assessed.

Materials and Methods

Study design and compliance

The HA integrating this study emerged from the consent of pets' owners attending the Veterinary Hospital of the

[1]Veterinary Clinics Department UPVET, Abel Salazar Institute for the Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal.
[2]Microbiology and Food Technology Laboratory, ICBAS, University of Porto, Porto, Portugal.
[3]Interdisciplinary Center for Marine and Environmental Research (CIIMAR), University of Porto, Porto, Portugal.

University of Porto (UPVet) in Portugal. Eligibility criterion for this study required that the core pet (the animal visiting the hospital), from the applicant domestic aggregate (that included owners and other pets), had been submitted to at least one antimicrobial treatment during the previous 6 months. The owners were asked to sign in a term of acceptance, to fill a questionnaire (about intrinsic and environmental variables of each of the HA elements, including human and veterinary medical records with antibiotic exposure), to bring their own stool samples, and to allow the collection of fecal, oral secretions' samples, and skin and fur swabs from their pets and also swabs from their own hands and from commonly touched household objects and surfaces (light switches, door knobs, TV remote control, mobile phones, banister, refrigerator door handle, kitchen floor, pets bed, leash, food and water recipients). Approval was obtained from the Ethical Committee of the Abel Salazar Institute for the Biomedical Sciences, University of Porto.

Enterococci isolation

Fecal samples were collected and immediately diluted 1:10 in Buffered Peptone Water (BPW; Oxoid) and stored at room temperature for 30 min. From the initial suspension, an aliquot of 5 ml was streaked on Slanetz and Bartley (SB) Agar (Biokar Diagnostics), and 100 ml were cultivated on three agar plates of SB supplemented with 8 mg/ml of ampicillin (AMP), 4 mg/ml of ciprofloxacin (CIP), or 6 mg/ml of vancomycin (VAN; Sigma-Aldrich), respectively. The swabs collected from surfaces and objects were immersed on BPW for 30 min at room temperature. Then, two sets of agar plates, supplemented or not with antibiotics (AMP, CIP and VAN), were inoculated with 100 ml from each sample.

Plates were incubated overnight at 37°C. Five colonies with typical appearance of enterococci were selected from the non-supplemented SB agar plates and all colonies presenting different morphologies were additionally picked from the supplemented plates.

Antimicrobial susceptibility characterization

Antimicrobial susceptibility testing of each isolate was carried out using the disk diffusion assay, following the guidelines provided by CLSI.[6] A total of 11 antimicrobial agents (Oxoid) were tested: AMP (25 mg), tetracycline (TET, 30 mg), rifampicin (RIF, 5 mg), gentamicin (GEN, 10 mg), chloramphenicol (CHL, 30 mg), CIP (5 mg), erythromycin (ERY, 15 mg), teicoplanin (TEC, 30 mg), VAN (30 mg) quinupristin/dalfopristin (QD, 15 mg), and nitrofurantoin (NIT, 300 mg). The antimicrobial drugs were selected to include those regularly used in both human and veterinary medicine and to provide diversity by representing different antimicrobial classes.

DNA extraction

The genomic DNA of Enterococcus spp. was extracted from bacterial cells grown overnight at 37°C in Brain Heart Infusion (Biokar Diagnostics), using an enzymatic treatment with 25 ml lysostaphin (1 mg/ml; Sigma-Aldrich) for 2 hr at 37°C, followed by treatment with 2.5 ml lysozyme (50 mg/ml; AppliChem GmbH) and 2.5 ml proteinase K (20 mg/ml; Bioron) for another 2 hr at the same temperature.

Species identification

A multiplex polymerase chain reaction (PCR) was performed for Enterococcus species identification. Amplification of the genes related to the species-specific identification of Enterococcus faecalis, Enterococcus faecium, Enterococcus gilvescens, Enterococcus durans, Enterococcus casseliflavus, Enterococcus gallinarum, Enterococcus avium, Enterococcus cecorum, Enterococcus hirae among other species was performed as described previously.[14]

Samples of PCR products were analyzed by electrophoresis in a 1.5% agarose gel (Seakem Agarose; Lonza), at 150 V for 45 min. Gels were stained with ethidium bromide (0.5 mg/ml) and photographed under UV light using a Molecular Imager Gel Doc XR (Bio-Rad Laboratories).

ERIC-PCR fingerprinting

Criteria designed for selecting the group of isolates, from each HA, eligible for genetic analyses were (i) multidrug-resistant enterococci with different antimicrobial resistance patterns and (ii) multidrug-resistant strains that had similar antimicrobial resistance phenotypes but were originated from different sources.

Ten microliters of the DNA extract was used in 25 ml ERIC-PCR mixture, containing 0.8 pmol/ml of each primer: ERIC-1R (5′-ATG TAA GCT CCT GGG GAT TCA C) and ERIC-2 (5′-AAG TAA GTG ACT GGG GTG AGC G), 1× buffer, 0.2 mM of each dNTP, 5 mM $MgCl_2$, and 2 U Taq DNA polymerase.[23] The PCR was performed using an initial incubation at 95°C for 7 min, followed by 35 cycles consisting of 95°C for 30 sec, 48°C for 1 min, and 65°C for 8 min. A final extension at 65°C for 16 min was programmed to complete the amplification.[25]

ERIC-PCR patterns of each isolate were visualized after electrophoresis in a 1.5% agarose gel (Seakem Agarose; Lonza) at 150 V for 45 min. Gels were stained and visualized as described above.

Data analysis

The antimicrobial susceptibility patterns were analyzed for descriptive purposes based on relative frequencies. The assortment of all isolates phenotypically characterized, from each HA, was examined for the number of antimicrobial resistance determinants as well as for repetitive resistance patterns. For each HA, a collection with all multidrug-resistant strains with different antimicrobial patterns plus all multidrug-resistant strains with similar patterns but originated from different sources was created. The compilation of these isolates was used to build up the genetic component of the present study.

Similarities in ERIC-PCR patterns were compared by means of the Dice similarity coefficient using the Fingerprinting DST Molecular Analist Software (Bio-Rad Laboratories). The dendrograms were constructed by the unweighted pair group method using averages (UPGMA), and an optimization of 1% and a position tolerance of 2.0% were applied. According to some authors,[4,27] a minimum similarity cutoff of 80–85% should be used to determine the genotype diversity using the ERIC-PCR technique. Herein, we have used a similarity cutoff of 94%.

Results

Description of HA

One of the three HA studied, HA A, allowed complete sampling (that included pets, owners, and home environment), whereas HA B and HA C did not allow the collection of household samples.

The HA A is composed of a core dog, a 14-year-old female Cocker Spaniel (Dog A1). It was a chronically ill dog that had been treated repeatedly with antimicrobials (Table 1).

Two more dogs belong to HA A: a 5-year-old female Cocker Spaniel (Dog A2) and a 2-year-old male Boxer (Dog A3), both healthy, which have just visited the veterinary services for regular consulting. They have never been ill and have never taken any antimicrobial drug. Their owners, a middle-age couple (Gentleman A and Lady A), were both healthy with no recent antimicrobial treatment. The family lived in a peripheral urban villa with a garden, where dogs used to play (dog walking in the street rarely happened). All dogs were active elements of the family with free access to all the rooms and items within the house. Dogs A2 and A3, when they were puppies, they used to have coprophagic habits. The three pets lived with the owners since birth and were fed only with canned dry food.

The core pet from HA B (Cat B) was a 4-year-old crossbreed male cat. He was fed with canned dry food. He has been suffering from recurrent urinary tract infections requiring multiple courses of antimicrobial therapy (Table 1). The cat owners (Gentleman B, Lady B, and Lady Daughter B) were a healthy family without any antimicrobial treatment history. The family lived in an apartment, in the outskirts of a city. The cat, which lived with the owners since birth, had never left the house and had free access to all rooms.

HA C consists of a 7-year-old core dog (Dog C) and a middle age couple (Gentleman C and Lady C). The family lived in an apartment, at the outskirts of the city. Dog C had a chronic, poorly controlled, allergic skin disease with recurrent pruritus and pyoderma that required frequent antimicrobial therapy, as shown in Table 1. It was walked outside the house, free, twice a day. The dog had no coprophagic habits, lived with the owners since birth, and was fed with canned dry food. Owners were healthy and had not received recent antimicrobial therapy.

Enterococcus isolation and phenotypic characterization

A total of 94 enterococcal isolates were collected from 30 of 35 sources within HA A. No isolate was recovered from Dog A2 food bowl, Dog A2 toy, Dog A3 skin, mobile phone, and toilet flush bottom. Antimicrobial susceptibility tests displayed four different phenotypic patterns; three of them were repeatedly found in isolates obtained from dogs, their owners, and some household samples. These isolates were further studied using ERIC-PCR (Fig. 1). The majority of these isolates were resistant to TET (87.2%) and RIF (67.0%). No resistance to AMP, VAN and TEC was observed.

From HA B, 21 isolates were recovered (6 from Cat B feces, 5 from Lady B feces, 5 from Gentleman B feces and 5 from Lady Daughter feces). The majority of these isolates were CIP-resistant (92%). No resistance was found against AMP, VAN, TEC, CHL, and NIT. Antimicrobial susceptibility tests displayed four different phenotypic patterns; one phenotype was found in the cat, lady and lady daughter feces.

Twelve and 3 isolates were identified from Dog C feces and mouth, respectively. Twenty-six more strains were recovered from Lady C (11 from feces and 5 from hands) and from Gentlemen C (7 from feces and 3 from hands). Six different antimicrobial patterns were found, with resistances to RIF, azithromycin, and TET being predominant. All isolates were susceptible to VAN.

Antimicrobial resistance patterns of isolates selected from HA A, B and C for genetic analysis are shown in Figures 1–3, respectively.

Enterococcus species identification and clonality

Isolates, from all HA, eligible for genetic analysis (n=59) were identified as being E. faecalis. The genetic relatedness among isolates from each HA was examined by ERIC-PCR, considered a method with a good discriminatory power for enterococci.[27] Figure 1 displays the similarity scrutiny of strains from HA A. By analyzing ERIC-PCR profiles of isolates harboring the antimicrobial resistance phenotype CIP

Table 1. Recent Antimicrobial Treatment Histories of Each Household Aggregate Core Pet

Animal	Antimicrobial	Protocol	Duration (weeks)	Ending date
Dog A1	Enrofloxacin	2.5 mg/kg, PO, BID	6	3 weeks ago
	Amoxicillin–clavulanic acid	20 mg/kg, PO, BID	2	3 months ago
Cat B	Gentamicin	8 mg/kg, SC, SID	2	2 months ago
	Penicillin G procaine	30 mg/kg, SC, SID	2	2 months ago
	Enrofloxacin	2.5 mg/kg, PO, BID	2	3 months ago
Dog C	Amikacin	15 mg/kg, SC, SID	4	3 weeks ago
	Enrofloxacin	2.5 mg/kg, PO, BID	2	7 weeks ago
	Cefadroxil	22 mg/kg, PO, BID	2	7 weeks ago
	Metronidazole	22 mg/kg, PO, BID	2	7 weeks ago
	Clindamycin	11 mg/kg, PO, BID	2	4 months ago

PO, per os; SC, subcutaneous; BID, each 12 hr; SID, each 24 hr.

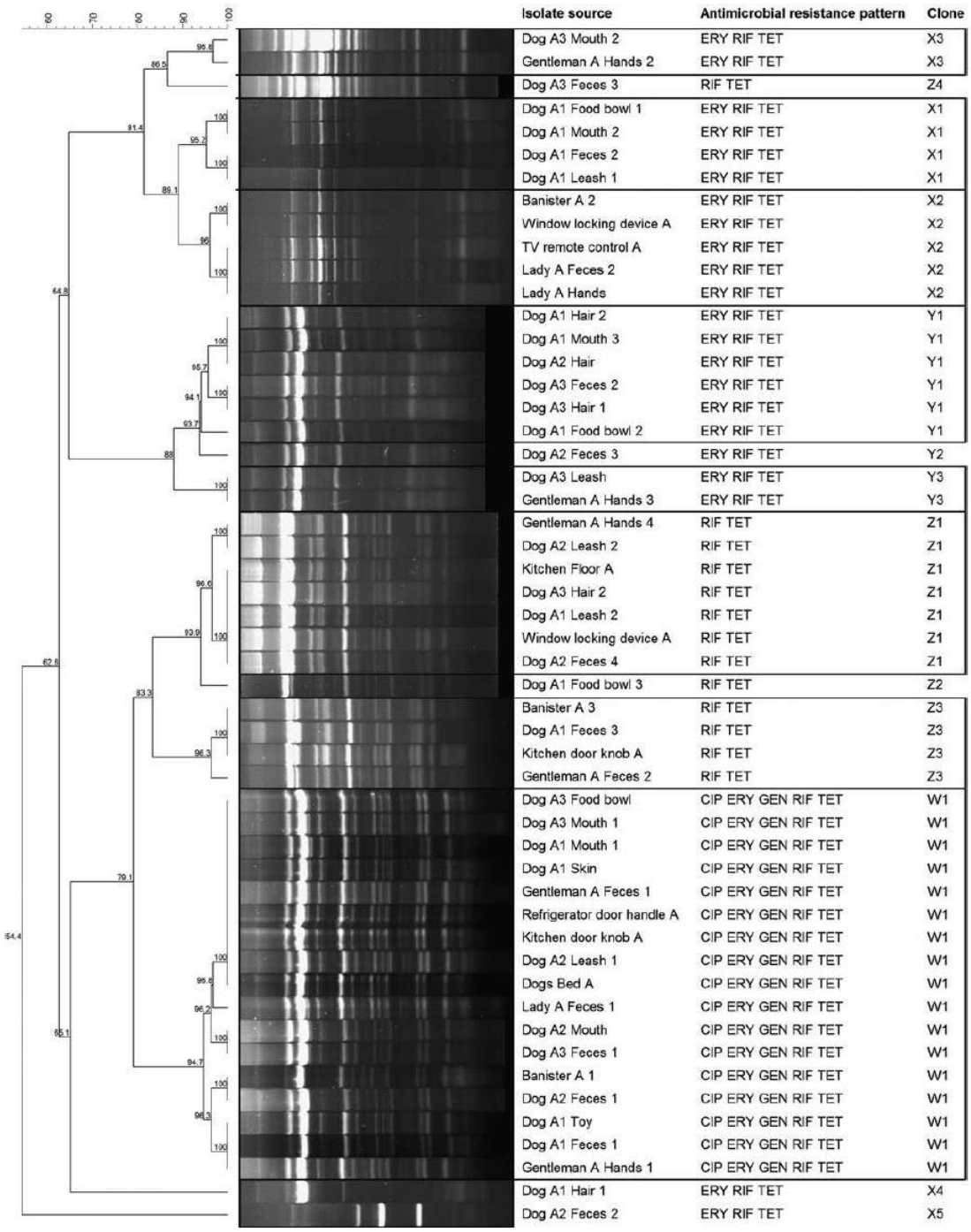

FIG. 1. Dendrogram showing genetic relatedness of selected isolates from HA A, determined by the analysis of ERIC-PCR fingerprint patterns using the Dice similarity coefficient and unweighted pair group method using averages (UPGMA) cluster method. HA, household aggregate.

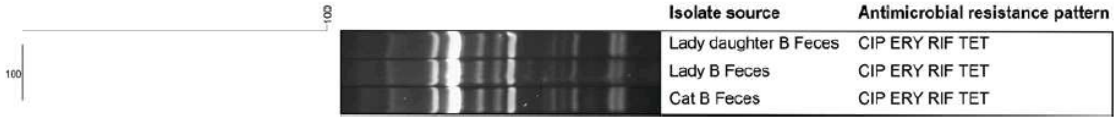

FIG. 2. Dendrogram showing genetic relatedness of selected isolates from HA B, determined by the analysis of ERIC-PCR fingerprint patterns using the Dice similarity coefficient and UPGMA cluster method.

ERY GEN RIF TET, we were able to classify these isolates as being the same clone W1 (94% similarity). In view of the same premises, the analysis of the other two phenotype groups from HA A, as shown in Figure 1, allowed the identification of seven main clonal spread situations (X1, 2, 3, Y1, Y3, Z1, and Z3), all of them composed of isolates from different sources (pets, humans, and home environment). In Figure 2, a 100% similarity between the strains is shown, suggesting an E. faecalis clonal spread between Cat B feces, Lady B feces, and Lady Daughter B feces. The same can be asserted for HA C, in which a clonal spread was perceived among Dog C mouth, Lady C hands, and Gentleman C hands, as shown in Figure 3.

Discussion

Some multidrug-resistant enterococci were isolated from all three HA studied.[17] Interestingly, the highest percentages of resistance were toward different antibiotics than the ones that were being taken by the core animals. The majority of resistance levels were observed for QD (whose resistance is intrinsic for E. faecalis), RIF, TET and ERY. No isolates showed resistance to VAN. CIP had been administered to all three core dogs; however, only in HA B, the majority of isolates were resistant to this antibiotic, whereas in HA A, only some isolates were CIP-resistant and no isolate collected from HA C was resistant to CIP. These observations are in agreement with previous reports stating that the use of fluoroquinolones may promote the resistance to other antimicrobial drugs.[16,19] The co-selection phenomena may explain this whole set of resistance mechanisms.[1]

Interestingly, in all three HA, enterococci that were selected for ERIC-PCR analyses were identified as being E. faecalis, suggesting that this species is becoming increasingly adapted to animals and is also capable of persisting and spreading within the household environment.

The constant repetion of antimicrobial resistance phenotypes of isolates recovered from very different sources was notable and led us to investigate these isolates further by genetic analysis. The results have revealed that the great majority of isolates sharing the same antimicrobial resistance phenotypes were found to have similar ERIC-PCR profiles (for a similarity 94%), suggesting that they were the same clone.

In HA A, intraspecies spread of E. faecalis, between core dog and respective dog cohabitants (owners), was detected as shown by the presence of similar antimicrobial resistance profiles and ERIC-PCR patterns in isolates recovered from them. Accordingly, a clonal spread between pets and their owners was also observed in all three HA, confirming interspecies dissemination. Furthermore, in HA A, the same clone that was present in pets and owners was also found in household surfaces and objects (banister, leash, toys, and door knobs) and disseminated over diverse body parts of the animal. In view of that, we can assume that within-household transmission[3] may occur, in either direction, mediated directly by feces, oral secretions, urine, skin, fur, or owners hands or indirectly by contaminated household surfaces and objects. In fact, such strains could be acquired by touching the contaminated surfaces or objects and be repeatedly transmitted between humans and animals, belonging to the household or even incoming visitors, building up the in-home and through-home transmission mode. Such possibilities confirmed concerns of the owners and were the driving reason for their study participation. Multidrug-resistant E. faecalis is potentially able to survive and persist outside the natural host long enough to have an opportunity to contaminate new hosts. Previous studies[8,12,21] have already described the presence of lineages of multidrug-resistant enterococci in dog's feces. VAN-resistant enterococci (VRE) were also found in feces of dogs and cats from urban areas[22] and are common in dogs from farms where VRE were also prevalent among other farm animals.[2,9]

In conclusion, in this study, the role of the household environment in the spread of common and multidrug-resistant enterococci to animal and human cohabitants was confirmed. Our results underline the importance of raising global awareness of the issue of antimicrobial resistance that is crossing species, clinical and international boundaries with remarkable speed.[5]

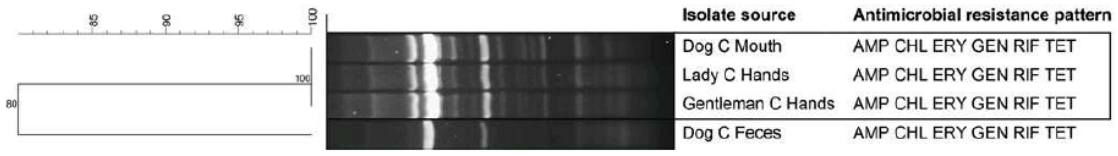

FIG. 3. Dendrogram showing genetic relatedness of selected isolates from HA C, determined by the analysis of ERIC-PCR fingerprint patterns using the Dice similarity coefficient and UPGMA cluster method.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Liliana Leite-Martins, DVM
Departamento de Cl□nicas Veterinarias
UPVET Cl□nica Veterinaria da Universidade do Porto
Instituto de Ciencias Biomedicas Abel Salazar
Universidade do Porto
Rua Jorge Viterbo Ferreira, 228
Porto 4050-313
Portugal

E-mail: lmartins@icbas.up.pt

Chapter 3

GENERAL DISCUSSION

FINAL REMARKS AND FUTURE PERSPECTIVES

3.1. GENERAL DISCUSSION

Besides the strategic purpose of veterinary medicine: “illness prevention”, small animal veterinary practitioners are frequently faced with the requirement for prescribing antimicrobial agents. Antimicrobial selection is a complex process involving several requests: the objective necessity for the treatment; possibility to obtain and to delay treatment until culture and sensitivity testing are performed; drug availability; drug cost, posology; toxicity; animal and owner compliance; adjuvant or alternative therapeutic options; and public health treatment implications. The complexity of managing some clinical cases submitted to several previous antimicrobial treatments, added to the veterinary duty of protecting the health of those persons that directly or indirectly contact with the treated animals, were the main reasons for this research project.

3.1.1. Antimicrobial resistance prevalence and risk factors - Papers I and II

Results from Papers I and II (Chapter 2) reflected the antimicrobial resistance prevalences in fecal *E. coli* and *Enterococcus* spp. isolated from domestic dogs and cats attending the Porto University Veterinary Hospital.

Regular surveillance of AMR in pathogens and normal flora has been recommended by the World Health Organization (WHO 2012b), although pet animals have not been usually included in such programs (Gosh *et al*., 2011). Recently, various research groups have studied and characterized the antimicrobial resistance arrangements of canine and feline *E. coli*, both commensal (Rantala *et al*., 2004; Moreno *et al*., 2008; Costa *et al.,* 2008a; Murphy *et al.,* 2009; Nam *et al.,* 2010; Leonard *et al*., 2012; Albrechtova *et al*., 2014; Okubo *et al.,* 2014) and pathogenic (Shaheen *et al*., 2010; Boothe *et al*., 2012; Nam *et al*., 2013; Wagner *et al.,* 2014) (Table 1).

Table 1. Overview of several studies on canine and feline *E. coli* antimicrobial resistances.

Number of sampled animals		Health status	Number of isolates	Sample period	Year	Country	Reference
Dogs	Cats						
33	-	33 UTI isolates	33	2002-2011	2014	UK	Wagner *et al.*
28	-	28 dogs	28	April-June 2003	2014	Japan	Okubo *et al.*
17	-	17 stray dogs	16	-	2014	Angola	Albrechtova *et al*.
10	-	10 UTI before tx	10	2008	2013	Korea	Nam *et al.*
301	75	Pathogenic isolates	376	May-September 2005	2012	USA	Boothe *et al.*
136	-	Healthy dogs	395	October 2005-May 2006	2012	Canada	Leonard *et al*.
877	-	565 Stray + 312 hospitalized	628	-	2010	Korea	Nam *et al.*
?	?	Pathogenic isolates	376	May-September 2005	2010	USA	Shaheen *et al*.
188	39	Healthy pets	1135	May-December 2002	2009	Canada	Murphy *et al.*
78	66	Healthy pets	144	2003	2008a	Portugal	Costa *et al.*
30	-	Hospital (52 tx + 18 non tx)	70	March-June 2006	2008	Chile	Moreno *et al.*
78	-	Hospital (22 tx Pyoderma + 56 non tx)	98	-	2004	Finland	Rantala *et al.*

Legend: UTI – urinary tract infection; USA – United States of America; UK – United Kingdom; tx – treatment.

Some studies aimed to illustrate the AMR profiles of fecal canine and feline commensal *E. coli* (Table 2). The comparison between their results is hampered by design variability, small number of studied animals, and lack of non-medicated control groups, the latter being often a consequence of low compliance on the animal owners part.

Table 2: Synopsis of antimicrobial resistance prevalence rates from canine and feline fecal *E. coli*.

Beta lactam								Aminoglicoside				Quinolone		Other				Reference
AMP	AMC	ATM	CEF	FOX	CAZ	CTX	IPM	GEN	STR	TOB	KAN	NAL	CIP	TET	CHL	SXT	NIT	
51.3	12.1	17.7	46.7	5.8	13.6	14.6	0	5.8	43.4	3.0	13.9	35.9	29.5	45.2	18.2	36.4	0	Leite-Martins *et al*. 2014
12.7	3.8	-	-	3.3	3.3[1]	3.3[1]	-	0.3	4.3	-	0.3	4.8	2.5	9.6	2.5	4.8	-	Leonard *et al*. 2012
32.9	5.2	-	8.5	4.7	-	2.4	0	-	35.8	16.1	-	21.6	13.5	53.6	17.1	19.7	-	Nam *et al*. 2010[2]
47.1	6.3	-	18.4	4.4	-	3.9	0	-	41.7	21.8	-	37.4	21.4	52.4	24.3	36.4	-	Nam *et al*. 2010[3]
13.0	-	-	13.0	-	-	-	-	-	17.0	-	-	-	-	11.0	-	-	-	Murphy *et al.* 2009[4]
4.0	-	-	1.0	-	-	-	-	-	2.0	-	-	-	-	2.0	-	-	-	Murphy *et al.* 2009[5]
12.0	3.5	1.4	-	0	0	1.4	0	2.1	15.0	15.0	-	3.5	0.7	20.0	2.8	-	-	Costa *et al.* 2008
12.0	-	-	-	-	-	-	-	-	12.0	-	-	-	-	10.0	-	8.0	-	Rantala *et al.* 2004[6]
18.0	-	-	-	-	-	-	-	-	22.0	-	-	-	-	0	-	15.0	-	Rantala *et al.* 2004[7]

Legend: AMP – ampicillin; AMC – amoxicillin-clavulanic acid; ATM – aztreonam; CEF – cephalothin; FOX – cephoxitin; CAZ – ceftazidime; CTX – cefotaxime; IPM – imipenem; GEN – gentamicin; STR – streptomycin; TOB – tobramycin; KAN – kanamycin; NAL – nalidixic acid; CIP – ciprofloxacin; TET – tetracycline; CHL – chloramphenicol; SXT – trimethoprim-sulfamethoxazol; NIT – nitrofurantoin; [1] – Different antimicrobials from the same family; [2] – Stray animals; [3] – Hospitalized animals; [4] – Dogs; [5] – Cats; [6] – Non-treated animals; [7] – Treated animals.

If studies that used antimicrobial supplemented medium for *E. coli* isolation (Moreno *et al*., 2008; Albrechtova *et al*., 2014; Okubo *et al*., 2014) were excluded from the analysis, it appears that the majority of the remaining ones reported lower AMR prevalences when compared with our results. Only one study (Nam *et al*., 2010) of 565 stray and 312 hospitalized dogs reported prevalences similar to ours. The authors hypothesized that such high AMR frequencies may have been a consequence of the inclusion of hospitalized animals as well as the high volumes of antimicrobials used by Korean veterinary practitioners.

Similarly, various studies aimed to characterize the antimicrobial resistance frequencies of canine and feline enterococci, both commensal (Rodrigues *et al*., 2002; Leener *et al*., 2005; Poeta *et al.,* 2006; Damborg *et al*., 2008; Ossiprandi *et al.,* 2008; Damborg *et al*., 2009; Jackon *et al*., 2009; Jackson *et al.,* 2010; Türkylmaz *et al*., 2010; Ghosh *et al*., 2011; Lopez *et al*., 2011; Hamilton *et al*., 2013; Kataoka *et al.,* 2013) and

pathogenic (Kwon *et al.,* 2011; Tremblay *et al*., 2013) (Table 3). The results of some studies are not comparable to ours because enterococci isolation was performed on antimicrobial supplemented media (Damborg *et al*., 2009; Jackson *et al*., 2010) or because the enterococci pool was mainly constituted by pathogenic isolates (Kwon *et al*., 2011; Tremblay *et al*., 2013). Data from the remaining studies are displayed in Table 4.

Table 3. Overview of studies on canine and feline enterococci antimicrobial resistances

Number of sampled animals		Health status	Number of isolates	Sample period	Year	Country	Reference
Dogs	Cats						
622	92	Healthy + Ill	1111	February 2007-December 2009	2013	Michigan	Hamilton *et al.*
5		Clinical ARE	5	?	2013	Canada	Tremblay *et al.*
60	31	Healthy + Ill	91	January-November 2006	2010	Turkey	Türkylmaz *et al.*
7	-	Under antimicrobial Tx in ICU	207	2008-2009	2011	Kansas	Ghosh *et al.*
?	-	28 UTI + 10 fecal	38	January 2010-May 2011	2011	Korea	Kwon *et al.*
126	-	Healthy + Ill	126	Jun-July 2009	2011	Spain	Lopez *et al.*
155	121	Healthy + Ill	420	2007	2010	USA	Jackson *et al.*
155	121	Healthy + Ill	420	2007	2009	USA	Jackson *et al.*
208	-	Health + Ill	208	2006 and 2007	2009	UK + Denmark	Damborg *et al.*
127	-	Healthy + Ill	73	Jun-August 2006	2008	Denmark	Damborg *et al.*
99	-	56 tx <6 months + 43 non tx	165	?	2006	Italy	Ossiprandi *et al.*
39	32	Healthy	142	2003	2006	Portugal	Poeta *et al.*
88	72	Healthy + Ill	201	2002-2003	2005	Belgium	Leener *et al.*
85	19	Healthy + Ill	104	?	2002	Portugal	Rodrigues *et al.*

Legend: ARE – ampicillin-resistant enterococci; ICU – intensive care unit; UTI – urinary tract infection; Tx – treatment.

Table 4: Synopsis of antimicrobial resistance prevalence rates from canine and feline fecal enterococci.

Glicopeptide			Macrolide		Other							Reference
QD	TEC	VAN	ERI	AZM	AMP	TET	RIF	GEN	CHL	CIP	NIT	
54.0	**2.2**	**1.0**	**53.0**	**58.4**	**12.1**	**67.0**	**60.3**	**6.3**	**6.3**	**29.5**	**9.2**	Leite-Martins *et al*., 2014
-	-	-	33.0	-	4.3	-	-	44.7	12.8	23.4	-	Kataoka *et al*. 2013 [3]
-	-	-	45.3	-	37.5	-	-	48.4	35.9	75.0	-	Kataoka *et al*. 2013 [4]
-	-	-	43.9	-	6.8	-	-	42.4	28.8	12.1	-	Kataoka *et al.* 2013 [5]
52.4	-	0.4	-	-	-	39.0	37.5	-	-	-	-	Hamilton *et al.* 2013
-	0	0	63.0	-	4.0	70.3	-	14.0	11.0	-	-	Türkyilmaz *et al.* 2010
NA*	-	0	57.0	-	5.0	60.0	-	-	-	32.0	0	Ghosh *et al.* 2011[1]
-	-	0	54.0	-	98.0	85.0	-	50.0	-	98.0	28.0	Ghosh *et al.* 2011[2]
-	-	-	10.7	-	10.2	51.4	-	5.7	4.7	2.4	4.5	Jackson *et al*., 2009
-	-	0	8.0	-	0.0	31.0	65.0	2.0	2.0	0	-	Damborg *et al.* 2008[1]
0	-	0	30.0	-	20.0	30.0	60.0	0	0	20.0	-	Damborg *et al.* 2008[2]
-	-	0	89.2	-	6.2	84.6	90.8	-	-	16.9	-	Ossiprandi *et al.* 2006[1]
-	-	0	84.6	-	25.0	84.6	69.2	-	-	57.7	-	Ossiprandi *et al.* 2006[2]
-	0	0	47.0	-	1.0	50.0	-	6.0	6.0	8.0	-	Poeta *et al*. 2006
2.0	-	0	26.0	-	-	41.0	-	-	11.0	-	-	Leener *et al.* 2005[6]
12.0	-	0	31.0	-	-	66.0	-	-	8.0	-	-	Leener *et al.* 2005[7]
4.0	-	0	15.0	-	-	38.0	-	-	4.0	-	-	Leener *et al*. 2005[8]
0	-	0	53.0	-	-	81.0	-	-	31.0	-	-	Leener *et al.* 2005[9]
15.0	-	0	40.0	-	-	75.0	-	-	5.0	-	-	Leener *et al.* 2005[10]
	-	0	100.0	-	21.2	95.2	-	-	-	71.3	-	Rodrigues *et al*. 2002

Legend: AMP – ampicillin; QD - Quinupristin/dalfopristin; TEC – teicoplanin; VAN – vancomycin; ERI - erythromycin; AZM - azithromycin; TET – tetracycline; RIF - rifampicin; GEN – gentamicin; CHL - chloramphenicol; CIP – ciprofloxacin; NIT – nitrofurantoin; [1] - *E. faecalis*; [2] - *E. faecium*; [3] - almost without antimicrobial exposure; [4] - with antimicrobial exposure; [5] - without antimicrobial exposure (puppies and kittens); [6] - Privately owned dogs; [7] - Kennel dogs; [8] - Privately owned cats; [9] - Cattery cats; [10] - Hospitalized cats; NA*: not applicable to *E. faecalis* isolates due to their intrinsic resistance.

In the work of Ghosh *et al.* (2011), a considerable higher AMR prevalence was found for ampicillin (98.0%), gentamicin (50.0%), ciprofloxacin (98.0%) and nitrofurantoin (28.0%) (Table 4). The authors postulated that it was a consequence of the recent antimicrobial selective pressures over commensals from ICU patients under antimicrobial treatment, potentiated by the enterococci ability to horizontally transfer their resistance traits. Similar results were obtained by Kataoka *et al.* (2013) that characterized the AMR of three enterococci groups with different antimicrobials exposure histories (almost without antimicrobial exposure; with antimicrobial exposure and puppies and kittens without antimicrobial exposure) and concluded that prior antimicrobial exposures had a significant impact on the resistance rates for ampicillin and ciprofloxacin (37.5% and 75.0%, respectively). Not only the prior antimicrobial exposure has a substantial influence in the acquisition of AMR (Rodrigues *et al.*, 2002; Leener *et al*., 2005; Damborg *et al.,* 2008) but the elapsed time from the last exposure is of paramount importance, as demonstrated by Ossiprandi *et al.* (2006).

In our studies, both microorganisms presented higher resistance frequencies than previously reported (Tables 2 and 4), with *E. coli* isolates being particularly illustrative of the phenomenon. Although it could be stated that the Porto city area follows the urban trend of higher pet longevity, better veterinary care and widespread use of antibiotics in companion animal treatments; there are no evidences that such characteristics are in any way different from other areas. However, it has been showed that the Porto region suffers from a high level of environmental contamination with multidrug resistant enterococci and *E. coli* (Novais *et al.,* 2005; Martins da Costa *et al.,* 2006; Simões *et al.,* 2010; Flores *et al.,* 2013; Varela *et al*., 2014). It seems plausible to assume that these resistance acquisitions are multifactorial and its mitigation is not possible with single or simple measures; still, with such worryingly high levels of resistance, it seems imperative that mitigating measures need to be urgently implemented. These may include:

i) Privilege the topical treatment of skin and ear diseases;

ii) Systematically culture and test for resistances of all suspected infectious diseases before using antimicrobial therapies. Even in emergency cases where antimicrobial treatments cannot be postponed, such routine allows for invaluable information in the guidance of future preventive measures;

iii) Favor, when prescribing antimicrobial drugs, those with higher bacterial fitness cost and, simultaneously, with resistances less prone to be maintained through co-selection with other antimicrobials still in clinical use. The magnitude of these

critical parameters is the main biological feature that influences the rate of development of resistance, the stability of the resistance and the rate at which the resistance might decrease if any prescribing reduction policy is adopted (van Elsas *et al.,* 2011; Perry and Wright, 2013).

iv) Instruct pet owners to invest on prophylaxis (*e.g.* vaccines, ectoparasiticides, preventive allergic skin and ear management, dental hygiene, early diagnosis);

v) Educate pet owners on the measures to reduce exposure to environmental sources of microorganisms (*e.g.* refrain from drinking untreated water from natural sources, do not feed the animals with row food, reduce the contact with other animals' feces, regular skin and coat hygiene).

In our studies the prevalence of resistant fecal enterococci and *E. coli* was associated with previous exposure to antimicrobials, corroborating previous reports (McEwen and Fedorka-Cray, 2002; Berge *et al*., 2006; Enne, 2010; da Costa *et al*., 2013). In the multivariable analysis two risk factors emerged as significantly associated with the presence of multiple antimicrobial resistance phenotypes in both bacterial species: "previous treatment with quinolones" and "coprophagic habits".

A quinolone selective pressure, enhancing the emergence of the respective resistant bacteria, whose resistance determinants are recognized for confering to microorganisms a low fitness-cost (Marcusson *et al*., 2009), is also known to induce resistance to other antibiotics through the genetic linkage between resistance genes (Andersson and Hughes, 2010). These facts support the concept that the use of quinolones should be restricted only to the least and indispensable situations. Furthermore, pets submitted to quinolone treatments should be handled properly and their hygiene reinforced, both during hospitalization (recommended) and at home, during and after treatment. All secretions, excrements and touched fomites should be considered contaminated with AMR bacteria and dealt accordingly.

Coprophagy promotes the inoculation of own or foreign, potentially antimicrobial resistant, enteric flora. Furthermore, some feces may contain high concentrations of antibiotics, particularly those with poor oral bioavailability, so their consumption may lead to drug transfer between animals (allocoprophagy) or drug recycling (autocoprophagy), enhancing the emergence and dissemination of AMR (Toutain *et al.,* 2010). Coprophagy was traditionally considered a minor inconvenience for both pet owners and veterinary

practitioners, mostly for being as a repugnant habit rather than a health risk. However, our results demonstrate that it may have serious public health consequences by favoring the emergence of AMR. Consequently, such habit must be reversed or prevented and the potential consequences of its persistence explained to owners.

3.1.2. Household antimicrobial resistance share and spread – Papers III, IV and V

In opposition to many other medical products, antimicrobial prescription to an individual may affect the health of others. This non-obvious dimension for antimicrobial use and our anthropocentric view of human pathogens led us to ignore for decades, the existence of an ecological cycle that allows resistant bacteria, selected by the administration of antibiotics in other domains, to colonize or transfer resistance genes to pathogenic and commensal human bacteria (Rodrigues *et al.,* 2002; Guardabassi *et al*., 2004; Leener *et al*., 2005; Damborg *et al.,* 2008; Ossiprandi *et al*., 2008; Ghosh *et al*., 2011; da Costa *et al*., 2013; EMA, 2013; Kataoka *et al*., 2013).

In the second part of this thesis, the role of pets in the dissemination of multidrug-resistant *E. coli* and *Enterococcus* spp. throughout its body surfaces, household environment and cohabitants was explored.

Related clones of both microorganisms (Papers III and IV for *E. coli* and Paper V for *Enterococcus* spp.) were identified:

i) In different body parts of each studied core pet (skin, oral secretions and fur);

ii) In their human (hands and feces) and animal (skin, oral secretions and fur) cohabitants;

iii) In various household surfaces and objects (door knobs, locking devices, banisters, refrigerator door handles, kitchen floors, pet beds, leashes, toys, food and water recipients).

These results support the concept of within household AMR transmission by intra and inter-species transfer of multidrug-resistant bacteria, and highlight the crucial role of the household environment as suitable for the spread of multidrug-resistant bacteria amongst its inhabitants. Such conclusion emphasizes the importance of preventive measures both at the hygienic and social/interactive levels in domestic aggregates that

include pets, especially if there are family members or pets that are or have been submitted to antimicrobial treatments.

Knowledge provided from these preliminary investigations contributes to raise the global awareness on the AMR problem, both in human and veterinary clinical settings and in the general community. In this perspective, the results herein obtained should:

i) Assist in the implementation of safer handling procedures outside the house, namely the importance of removing the pets' feces from public areas, hence reducing the chance for coprophagic habits and the spread of AMR microorganisms and antibiotic substances in the environment;

ii) Assist in the implementation of safer handling procedures inside the house, namely the improvement of hygienic habits towards pets, objects and facilities, particularly when animals or owners are submitted to antimicrobial treatments. Such procedures must be implemented and enforced by veterinary practitioners as part of the recommendations on the antibiotics usage;

iii) Scientifically support veterinary practitioners to assume a more cautious and responsible attitude when prescribing antimicrobials and to contribute for the creation of guidelines for safe antimicrobial prescription, administration and handling.

iv) Justify the implementation of in-hospital biosafety rules and practices, as well as a clear definition of drugs that require hospitalization of the patient in order to be safely administrated.

During the past two decades, AMR studies have been conducted mainly in food-producing animals. As a consequence, these species are now considered as an important part of the global cycle of enrichment and dissemination of AMR species (CDC, 2013). Our results, albeit preliminary, point to the need of regularly and systematically monitor AMR in companion pets and in the domestic biome.

3.2. FINAL REMARKS AND FUTURE PERSPECTIVES

The results of the present study must be regarded as preliminary and a starting point for the collection of information about the prevalence, incidence, and risk factors for AMR in companion animals, as well as its public health impact. Such knowledge is essential for the implementation of effective rules and practices by all professionals involved in the protection of public health, such as veterinarians, human health professionals, pharmacists, animal breeders, handlers, and trainers.

Within this perspective, future studies on this important issue are warranted, namely in order to:

i) Improve monitoring studies with larger sampled animals; the specific study of particular groups such as cats, young animals, health professionals owned animals, animals under anti-tumor chemotherapy; more extensive anamnestic data such as the particularities of the antibiotic therapeutic regimens, hospitalizations and previous illnesses;

ii) Expand survey studies to the monitoring of the AMR decline in order to better characterize the fitness cost as well as the co-selection persistence and magnitude for the different antimicrobial classes;

iii) Perform more and extensive household researches to better understand and explore critical points at the household biome;

iv) Produce, analyze and divulgate data in order to perform some well justified guidelines on safer and improved antimicrobial prescription protocols as well as on ideal handling and petting in-treatment animals.

Chapter 4

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4. REFERENCES

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Chapter 5

ANNEXES

5. ANEXOS

Outros trabalhos científicos motivados pela presente Tese

Artigo em revista de circulação internacional com arbitragem científica

Beça, N.M., Bessa, L., Mendes, A., Santos, J., Leite-Martins, L., Matos, A., Martins da Costa, P. (2014). Coagulase-positive *Staphylococcus* - prevalence and antimicrobial resistance in companion animals, veterinary professionals and clinical environment. *Journal of the American Animal Hospital Association*. (*In press*).

Artigo em revista de circulação nacional com arbitragem científica

Martins da Costa, P., Leite-Martins, L., Antunes, F., Simões, R. (2010). Transferência de bactérias resistentes aos antimicrobianos entre nichos ecológicos interligados: homem, animais e ambiente. *Revista da Faculdade de Medicina de Lisboa*, série III 15 (5/6): 319-326.

Publicações em atas de encontros científicos

Comunicações Orais

Leite-Martins, L. (2013). Prevalência da resistência aos antimicrobianos em *Escherichia coli* e *enterococcus* spp. isolados em cães e gatos e estudo dos respetivos fatores de risco. *VIII Congresso OMV*. Lisboa, Portugal. 30 de Novembro a 01 de Dezembro de 2013.

Martins da Costa, P., Simões, R., Martins, L., Matos, A.J. (2011). O ciclo ambiental das resistências antimicrobianas (*Environmental dissemination of drug-resistant bacteria between intermingled ecological niches*). *V Congresso de Ciências Veterinárias 2011*. Sociedade Portuguesa de Ciências Veterinárias. Santarém, Portugal. 14 de Outubro de 2011, (Pp.57).

Comunicações Posters

Meireles, D.M., Martins, L.R., Bessa, L.J., Mendes, Â.J., Cunha, S.A., Matos, A., da Costa, P.M. (2014). Estudo da partilha de clones bacterianos entre animais de companhia, coabitantes humanos e superfícies domésticas. *VI Congresso da Sociedade Portuguesa de Ciências Veterinárias*: Praxis e futuro, Oeiras, Portugal, 3-5 de Abril. (Pp.127).

Leite-Martins, L**.**, Beça, N., Lopes, E., Frias, C., Matos, A., Martins da Costa, P. (2012). In-home and through-home transmission of antimicrobial resistance between human and pets. *II International Conference on Antimicrobial Research – ICAR 2012*, Lisbon, Portugal, 21-23 November. (Pp:410).

Beça, N.M., Simões, R.L., Santos, J.C., Lopes, E., Leite-Martins, L., Matos, A., Martins da Costa, P. (2012). Culture media isolation of *Staphylococcus pseudointermedius* and *Staphylococcus* spp. coagulase positive prevalence in domestic animals, Veterinary practitioners, Veterinary auxiliary workers and environment of a Veterinary hospital. *II International Conference on Antimicrobial Research – ICAR 2012*, Lisbon, Portugal, 21-23 November. (Pp:387).

Artigo em revista de circulação internacional com arbitragem científica

COAGULASE-POSITIVE *STAPHYLOCOCCUS* - PREVALENCE AND ANTIMICROBIAL RESISTANCE IN COMPANION ANIMALS, VETERINARY PROFESSIONALS AND CLINICAL ENVIRONMENT

Beça, N.M., Bessa, L., Mendes, A., Santos, J., Leite-Martins, L., Matos, A., Martins da Costa, P.

Journal of the American Animal Hospital Association. 2014. (*In press*).

Coagulase-positive *Staphylococcus* – prevalence and antimicrobial resistance in companion animals, veterinary professionals and clinical environment

Nuno Beça [a], Lucinda J. Bessa [a, c], Ângelo Mendes [a], Joana Santos [b], Liliana Leite-Martins [a, b], Augusto J. F. Matos [b], Paulo Martins da Costa [a]

[a] Microbiology and Food Technology Department, Abel Salazar Institute for the Biomedical Sciences (ICBAS), Porto University (UP), Portugal

[b] Veterinary Clinics Department, Abel Salazar Institute for the Biomedical Sciences (ICBAS), Porto University (UP), Portugal

[c] Interdisciplinary Center for Marine and Environmental Research (CIIMAR), University of Porto, Porto, Portugal

Abstract

Staphylococcus pseudintermedius is the most prevalent coagulase-positive *Staphylococcus* inhabitant of the skin and mucosa of dogs and cats, causing skin and soft tissue infections in these animals. In this study, coagulase-positive *Staphylococcus* species were isolated from companion animals, veterinary professionals and objects of a clinical veterinary environment, by using two particular culture media, Baird-Parker RPF agar and CHROMagar Staph aureus. Different morphology features of colonies on the media allowed the identification of the species, which was confirmed by performing a multiplex PCR. Among 23 animals, 15 (65.2%) harbored coagulase-positive *Staphylococcus*, being 12 *Staphylococcus pseudintermedius* carriers. Four out of 12 were methicillin-resistant *S. pseudintermedius* (MRSP). All veterinary professionals had CoPS species on their hands and two out of nine objects sampled harbored MRSP. The antimicrobial resistance pattern was achieved for all isolates, revealing the presence of many multidrug-resistant CoPS, particularly *S. pseudintermedius*. The combined analysis of the antimicrobial resistance patterns shown by the isolates led to the hypothesis that there is a possible cross contamination and dissemination of *S. aureus* and *S. pseudintermedius* species between the three types of carriers sampled in this study that could facilitate the spread of the methicillin resistance phenotype.

1. Introduction

Coagulase-positive *Staphylococcus* (CoPS) species are commensal bacteria present in skin and nasal flora, however they can cause opportunistic infections in animals and humans (Devriese *et al*., 2005; Sasaki *et al*., 2010). Among CoPS, *Staphylococcus pseudintermedius* has particular importance in the veterinary setting, mainly in small animals, being associated with dermatological problems such as pyoderma, post-operatory wound infection and otitis (Griffeth *et al*., 2006; Weese and Duijkeren 2010; Sasaki *et al*., 2010). Although, the zoonotic potential of *S. pseudintermedius* is not well defined yet, it has been isolated from human infections and humans in contact with animals (van Duijkeren *et al*., 2008; Paul *et al*., 2011; van Duijkeren *et al*., 2011). In addition, since the phenotypic differentiation of CoPS species is difficult, it is probable that *S. pseudintermedius* has been misidentified in routine laboratory diagnostics with other CoPS, especially *Staphylococcus intermedius* and *Staphylococcus aureus*, and thus its prevalence may have been underestimated (Weese and Duijkeren 2010).

Several methods to isolate and identify *S. pseudintermedius* have been documented. A combination of biochemical tests (D-mannitol test, arginine dihydrolase test and β-gentibiose test) are particularly used to phenotypically differentiate other *Staphylococcus* species from *S. pseudintermedius* (EMA, 2013). Molecular methods such as Pulsed-Field Gel Electrophoresis (PFGE), multiplex-PCR and PCR-restriction fragment length polymorphism are the most effective for *S. pseudinterm*edius identification (van Duijkeren *et al*., 2008; Sasaki *et al*., 2010; Bannoehr *et al*., 2009).

Similarly to *S. aureus*, *S. pseudintermedius* can acquire the *mecA* gene, which is located on staphylococcal cassette chromosome *mec* (SCC*mec*) elements and confers resistance to β-lactam antibiotics by encoding an altered penicillin binding protein (Perreten *et al*., 2010). The number of cases reporting methicillin-resistant *S. pseudintermedius* (MRSP) has been increasing and, usually, these MRSP are multidrug-resistant (van Duijkeren *et al*., 2008; Bannoehr *et al*., 2009; Kadlec *et al*., 2010; Perreten *et al*., 2010; Weese and Duijkeren 2010; Gómez-Sanz *et al*., 2011; Paul et al., 2011; van Duijkeren *et al*., 2011; EMA, 2013;).

The phenotypic identification of MRSP species can be made by antimicrobial susceptibility testing using a MIC breakpoint for oxacillin of ≥ 0.5 mg/l in broth dilution methods or by measuring an inhibition halo with a diameter ≤ 17 mm when using 1μg of oxacillin/disc in the agar diffusion methods, following the interpretative criteria of CLSI

(formerly NCCLS) of 2004 (NCCLS, 2004; Bemis *et al*., 2009; Schissler *et al*., 2009). Both tests can be highly consistent to detect MRSP species, however the detection of *mecA* gene by PCR is still the most reliable and also confirmatory method to identify methicillin-resistance *Staphylococcus* species (Black *et al*., 2009; Perreten *et al*., 2010; Ruscher *et al.,* 2010).

The main objective of this study was to find a new isolation method that could differentiate *S. pseudintermedius* from other CoPS using culture media. Two agar media were used, the Baird-Parker RPF agar, which has been mostly used in Food Microbiology for the direct detection and enumeration of coagulase-positive Staphylococci and CHROMagar Staph aureus, which is a selective medium for the isolation, enumeration and identification of *S. aureus* from clinical and food sources. Subsequently, the prevalence of two CoPS species, *S. pseudintermedius* and *S. aureus*, isolated from domestic animals, veterinary professionals and environment of a veterinary hospital was achieved. The antimicrobial resistance profile of CoPS isolates was also determined. Finally, there was an attempt to establish a possible correlation between all collected samples.

2. Materials and Methods

2.1. Sampling

Companion animals

Between February and May 2013, a total of 23 animals (21 dogs and two cats) were enrolled at the Veterinary Hospital of XXX after the positive consent of the owners, in order to collect samples from skin and oral and nasal mucosae. Two samples were collected from each body site, soon after the animal observation by a veterinarian, using a pre-moistened sterile swab and inoculated in 5 ml of Brain Heart Infusion[1] (BHI) supplemented with 0.1 % Tween 80[2] (T80) – BHI+T80. During the sample collection procedure, an inquiry was made to the owners, in order to obtain some information about potential risk factors for the presence of *S. aureus* or *S. pseudintermedius*, such as animal age, sex, residential area and animal health status.

[1] Oxoid, Basingstoke, United Kingdom
[2] Merck, Darmstadt, Germany

Veterinary professionals

A total of nine veterinary professionals, including veterinarians, technicians and veterinary nurses, affiliated with the Veterinary Hospital of XXX were recruited and consented samples collection, at the same day, for the isolation of *S. aureus* and *S. pseudintermedius*, from hands and nasal mucosa. The hand sample was collected with moistened sterile gauze and the nasal sample with two sterile swabs. Gauze and swabs were then placed in 50 ml and 5 ml of BHI+T80, respectively.

Clinical environment

On a single day, samples were collected from nine different objects and surfaces (e.g. floors, top parts of medical examination stands, computer keyboard, cages) of the veterinary hospital environment. There was no information about the disinfection status of the objects/surfaces. Sample collection was done with sterile gauze that was placed in 50 ml of BHI+T80. Afterwards, 1 ml was taken to perform a 1:10 dilution in 9 ml of BHI+T80. All samples were kept in the broth medium no longer than 1 hr until processing in the laboratory.

2.2. Bacterial isolates and antimicrobial susceptibility testing

The following procedure was similarly performed for all samples (animals, veterinary professionals and clinical environment samples).

At the laboratory, each sample was incubated at 37 °C. After 6 hr of incubation, an aliquot of 30 µl was inoculated onto Baird-Parker RPF.[3] Completed 18 hr of incubation, an aliquot of 30 µl was streaked onto Baird-Parker RPF and 60 µl were spread on the same culture media supplemented with oxacillin[4] (2 µg/ml). All plates were incubated at 37 °C for further evaluation of coagulase-positive activity at 24, 28, 32 and 48 hr.

During the observation period, every Baird-Parker RPF plate presenting typical coagulase positive colonies (white halo surrounding a well delimited round shape colony, whose color vary from grey to black) was subcultured by streaking onto CHROMagar™

[3] Biokar Diagnostics, Beauvais, France
[4] Sigma–Aldrich, St. Louis, MO

Staph aureus.[5] Two to five colonies isolated from each sampling site were selected for subculture. CHROMagar Staph aureus plates were incubated at 37 °C for 24 hr. After that time, a maximum of four colonies exhibiting typical *S. aureus* morphology (mauve-colored colonies) or *S. pseudintermedius* (purple and blue colonies) were selected for antimicrobial susceptibility testing and storage. Antimicrobial susceptibility testing was performed by the agar disc diffusion method on Mueller-Hinton agar, following the Clinical and Laboratory Standards Institute guidelines and interpretative criteria (formerly NCCLS) of 2004 (NCCLS, 2004), according to a previous study (Schissler et al., 2009), for a panel of 23 antimicrobial agents:[6] fucsidic acid (FD, 10 µg), amoxicilin (AMC, 10 µg), ampicillin (AMP, 10 µg), kanamycin (K, 30 µg), cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 µg), clindamycin (DA, 2 µg), chloramphenicol (C, 30 µg), erythromycin (E, 15 µg), streptomycin (S, 10 µg), gentamicin (CN, 10 µg), imipenem (IPM, 10 µg), lomefloxacin (LOM, 10 µg), neomicin (N, 10 µg), nitrofurantoin (F, 300 µg), oxacillin (OX, 1 µg), penicillin (P, 10 µg), quinupristin-dalfopristin (QD, 15 µg), rifampicin (RD, 5 µg), teicoplanin (TEC, 30 µg), tetracycline (TE, 30µg), trimethoprim/sulfamethoxazole (SXT, 25 µg) and vancomycin (VA, 30 µg). *Staphylococcus aureus* ATCC 25293 was used as a quality control strain.

2.3. **Species identification by Polymerase Chain Reaction (PCR)**

The DNA was extracted from isolated colonies that presented coagulase-positive activity in Baird-Parker RPF and the mauve, dark mauve, purple and blue colors in CHROMagar Staph aureus. A total of 41 DNA extractions were performed using lysostaphin[d] (100 µg/ml) and proteinase K[7] (100 µg/ml). Then, a multiplex PCR for the species-specific detection of *nuc* gene was performed by using the primers as previously described for the identification of three species of coagulase-positive staphylococci: *S. pseudintermedius*, *S. aureus* and *S. intermedius* (Sasaki *et al*., 2010). The reaction mixture for the PCR, with a total volume of 50 µl, consisted of 33 µl of distilled water, 5 µl reaction buffer (x10)-complete II KCl,[g] 1 µl of dNTP Mix 10 mM,[8] 2,5 µl of each *primer*, 1µl of *DFS-Taq DNA Polymerase* 500 U[g] and 5 µl of DNA. The reaction mixture was performed in a MyCycler Thermal Cycler[9] at 95°C for 1 min, followed by 30 cycles at 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7

[5] CHROMagar, Paris, France
[6] Oxoid, Basingstoke, United Kingdom
[7] Bioron GmbH, Germany
[8] Fermentas, Vilnius, Lithuania
[9] Bio-Rad Laboratories, Hercules, CA

min. Samples (5 µl) of PCR products were analyzed by electrophoresis in a 1.5% (w/v) agarose gel at 100 V for 60 min. Gels were stained with ethidium bromide and observed in a Ultra Violet Gel Doc XR.[i]

3. Results

Overall, 138, 18 and 9 samples were collected from companion animals (dogs and cats), veterinary professionals and objects/surfaces of the clinical environment, respectively. *Staphylococcus aureus* and *S. pseudintermedius* were phenotypically detected in the media Baird-Parker RPF and CHROMagar Staph aureus and genotypically confirmed by multiplex PCR. *Staphylococcus pseudintermedius* presented a white color in Baird-Parker RPF, with creamy consistence. Its coagulase halo was not as exuberant as the one presented by *S. aureus* colonies, which showed black to grey color with pasty consistence. In CHROMagar Staph aureus the main observation was the different color presented by *S. pseudintermedius* and *S. aureus* colonies. The first ones presented a color between purple and blue with aqueous consistency while *S. aureus* colonies presented mauve to dark mauve color with mucous consistency (see supplementary Figure I). The use of CHROMagar Staph aureus was particularly useful in one oral sample to allow the differentiation of colonies that appeared to be one single *Staphylococcus* species in Baird-Parker RPF. For every sample analyzed, each purple and blue colony in CHROMagar was identified by PCR (data not shown) as being *S. pseudintermedius* and the mauve and dark mauve colonies were identified as *S. aureus* species. The multiplex PCR confirmed not only the species identification as well as the "purity" of the colonies with different colors. For each colony tested, the presence of one *Staphylococcus* species excluded the presence of the other.

During the coagulase activity observation on Baird-Parker RPF, it was found that *S. pseudintermedius* presented a coagulase-positive activity only after 28 hr of incubation at 37 ^{o} C, instead of the 24 hr needed for *S. aureus* isolates to show the positive activity.

3.1. Prevalence of CoPS isolates in companion animals

Among the 23 animals, 15 (65.2%) had CoPS species and the remaining eight were non-CoPS carriers; 14 (93.3 %) out of the 15 were dogs, only one was a cat (6.7%) The distribution of the two CoPS isolated from the two kinds of animals and among different body sites of the animals is shown in Table 1. A detailed analysis of these 15 CoPS-animals allowed us to observe that eight (53.3%) were *S. pseudintermedius* exclusive carriers, three (20.0%) were *S. aureus* exclusive carriers and four (26.7%) of them carried both *S. aureus* and *S. pseudintermedius*. The oral mucosa was the site where *S. aureus* was most isolated. Two out of the seven *S. aureus* carriers were MRSA. Regarding *S. pseudintermedius*, it was similarly present in the three body sites sampled, being the skin the site that provided the major number of *S. pseudintermedius* isolates (Table 1). Moreover, it must be highlighted that from the 12 *S. pseudintermedius* carriers, four harbored MRSP. The antimicrobial resistance exhibited by the two *Staphylococcus* species isolated from the animals is presented on Table 2, showing multidrug-resistance particularly by *S. pseudintermedius* isolates. Diverse antimicrobial resistance patterns were shown by *S. pseudintermedius* and *S. aureus* isolated from different body sites of the animals (Table 3).

Table 1. Number of total isolates of *S. pseudintermedius* and *S. aureus* recovered per animal and per body site of the animal.

	Number of *S. pseudintermedius* isolates n(%)	**Number of *S. aureus* isolates** n (%)
Amimal		
Dog (n=14)	11 (73.3)	7 (46.7)
Cat (n=1)	1 (6.7)	0 (0.0)
Body site		
Oral mucosa	6 (50.0)	5 (71.4)
Nasal mucosa	5 (41.7)	3 (42.9)
Skin	7 (58.3)	0 (0.0)

Tabela 2. Antimicrobial resistance of all *S. pseudintermedius* and *S. aureus* isolated from animals.

Antimicrobial drug	***S. pseudintermedius*** (n = 12) n (%)	***S. aureus*** (n = 7) n (%)
Fucsidic acid (FD)	1 (8)	0
Amoxicilin (AMC)	3 (25)	1 (14.3)
Ampicillin (AMP)	12 (100)	5 (71.4)
Kanamycin (KAN)	6 (50)	1 (14.3)
Cefoxitin (FOX)	0	1 (14.3)
Ciprofloxacin (CIP)	4 (33.3)	1 (14.3)
Clindamycin (DA)	6 (50)	1 (14.3)
Chloramphenicol (CHL)	4 (33.3)	2 (28.6)
Erythromycin (ERI)	6 (50)	1 (14.3)
Streptomycin (STR)	6 (50)	1 (14.3)
Gentamicin (GEN)	3 (25)	0
Imipenem (IPM)	0	0
Lomefloxacin (LOM)	4 (33.3)	1 (14.3)
Neomicin (N)	6 (50)	1 (14.3)
Nitrofurantoin (NIT)	0	0
Oxacillin (OX)	4 (33.3)	2 (28.6)
Penicillin (P)	11 (91.7)	5 (71.4)
Quinupristin-dalfopristin (QD)	0	0
Rifampicin (RIF)	1 (8)	0
Teicoplanin (TEC)	0	0
Tetracycline (TE)	3 (25)	1 (14.3)
Sulfamethoxazole/trimethoprim (SXT)	4 (33.3)	0
Vancomycin (VAN)	0	0

Table 3. Antimicrobial resistance pattern of CoSP species isolated from different body sites of the animals.

Site of isolation	Isolated species	Antimicrobial* resistance profile	Animal identification
Oral mucosa	*S. aureus*	AMP P	17,22
		AMP S C K N E P DA	12
	S. pseudintermedius	AMP P	14,22
		AMP P OX CIP LOM	15
		AMP SXT OX CIP LOM	15
		AMP S C K N E P DA	6, 11
		AMP SXT P OX CIP LOM	15
		AMP SXT S AMC OX CIP K N E LOM P DA	13
		AMP SXT S AMC OX CIP C K N CN E LOM P DA	13
Nasal mucosa	*S. aureus*	AMP P	4
		AMP P TE OX C	17
	S. pseudintermedius	AMP P	21
		AMP S C K N E P DA	6,11
		AMP SXT S AMC OX CIP K N E LOM P DA	13
		AMP SXT P AMC OX CIP C K DA CN LOM S N	17
Skin	*S. aureus*		
	S. pseudintermedius	AMP P	20
		AMP SXT OX CIP LOM	15
		AMP S C K N E P DA	11,12
		AMP SXT RD P OX CIP LOM	15
		AMP SXT RD P OX FD CIP LOM	15
		AMP SXT P AMC OX CIP C K DA CN E LOM S N	17
		AMP SXT S TE AMC OXA CIP K N CN E LOM P DA	1,13

*For abbreviations: see Table 2

3.2. Prevalence of CoPS in veterinary professionals

The analysis of the samples collected from the veterinary professionals showed that all of them had CoPS species in their hands. However, only two presented CoPS in the nasal mucosa and were identified as being *S. aureus*. Eight (88.8%) *S. aureus* and five (55.6%) *S. pseudintermedius* were isolated from nine hand samples. All the *S.*

aureus were methicillin-sensitive (MSSA) and only one of the *S. pseudintermedius* was MRSP. The resistance pattern of the isolates is shown in Table 4.

Table 4. Antimicrobial resistance pattern of CoSP species isolated from 2 body sites of the veterinary professionals.

Site of isolation	Isolated species	Antimicrobial* Resistance profile	Number identifying the Professional
Hand	*S. aureus*	AMP P	1, 2, 3, 4, 5, 6, 7, 9, 9
	S. pseudintermedius	AMP P AMP P OX	1, 5, 6, 7, 8 7
Nasal mucosa	*S. aureus*	AMP P	2, 4
	S. pseudintermedius		

*For abbreviations: see Table 2

3.3. Prevalence of CoPS in objects and surfaces of the veterinary hospital

Only three (33.3%) out of the nine samples collected from the veterinary objects harbored CoPS. Two were *S. pseudintermedius* and both methicilin-resistant. *Staphylococcus aureus* was isolated only in one object and was a MSSA. In particular, one MRSP isolate showed resistance to a high number of antimicrobials (Table 5).

Table 5. Antimicrobial resistance pattern of CoSP species isolated from a surface and an object of the veterinary hospital.

Objects harboring CoPS	Isolated species	Antimicrobial* resistance profile
Cage floor	*S. pseudintermedius*	AMP SXT P AMC OX CIP K DA E LOM
	S. pseudintermedius	AMP SXT P AMC OX CIP K DA CN E LOM
Computer keyboard	*S. aureus*	AMP P

*For abbreviations: see Table 2

4. Discussion

Taking into account the present results, it may be appropriate to draw attention to the potential use of CHROMagar Staph aureus as a very selective medium to isolate not only *S. aureus* but also *S. pseudintermedius*. This medium can overcome other culture media such as Mannitol-Salt agar and Blood Agar, due to its selectivity for CoPS species and by hampering the proliferation of contaminant bacteria (Simões et al., 2011; van Duijkeren *et al*., 2011). The origin of color differentiation between *S. aureus* and *S. pseudintermedius* colonies on CHROMagar Staph aureus remains uncertain, however, it is probably related to the chromogenic mixture mentioned by the manufacturer or to the pH indicator present in this culture media. Though this medium appears to be reliable for *S. aureus* and *S. pseudintermedius* identification, molecular methods such as PCR or PFGE should always be recommended as confirmatory methods.

Regarding the prevalence of CoPS in the companion animals, it was observed that *S. pseudintermedius* isolates prevailed over *S. aureus*, which is not surprising (Hanselman *et al*., 2009; van Duijkeren *et al*., 2011). *Staphylococcus aureus* was mostly isolated from oral mucosa whereas *S. pseudintermedius* was equally present in the three body sites sampled. In fact, this finding is not in agreement with other reports, which stated that nasal and anal regions were the body sites more commonly colonized by *S. pseudintermedius* (Weese and van Duijkeren, 2010; Bannoehr and Guardabassi, 2012). It is also important to refer that *S. pseudintermedius* with different resistance profiles were isolated from the same animal and from the same sampled site.

All *S. pseudintermedius* were multidrug-resistant, showing resistance toward at least two antimicrobial agents, which is in accordance with previous observations (Ruscher et al., 2010; Stegmann et al., 2010; Detwiler et al., 2013). Moreover, four out of 12 *S. pseudintermedius* isolated from the animals were MRSP. The high number of MRSP found in the companion animals may be related to a regular use of antimicrobial agents to treat these animals. Available data indicates that in Portugal the use of antimicrobial agents in animals, including the use of drugs that are critically important to human medicine, is one of the highest amongst 19 European countries (EMA, 2013) unfortunately, there is no detailed information regarding the use of antimicrobials in companion animals. The growing number of household pets and their increasing health care standards led to an augmented number of geriatric animals, which have an extensive medical history, including antimicrobial drug administration, and longer contact with owners, increasing both the risk of antimicrobial resistance emergence and inter-species clonal spread.

In our work, the percentage of MRSP doubles the one of MRSA, supporting previous results (Ruscher *et al*., 2009) and represents an additional concern to the European efforts that are already trying to combat the spread of MRSA (EFSA, 2013). The close contact of small animals with people and also with other animals can promote the spread of resistant clones, namely methicillin-resistant clones, and may explain the increasing of MRSP species in small animals, even in healthy ones (Frank *et al*., 2009; Ruscher *et al*., 2010). A recent study that has screened healthy dogs in Portugal for the presence of nasal MRSA, concluded that those dogs may be a reservoir of MRSA that could be transmitted to humans, by direct contact (skin and mouth) or indirectly, via the household environment (Coelho *et al*., 2011). Thus, the high number of MRSA isolated from healthy dogs may also contribute to the disquieting scenario of MRSA in Portugal (according to the European Centre for Disease Prevention and Control, the proportion of MRSA amongst *S. aureus* clinical isolates in Portugal in the year 2011 was higher than 50%) (ECDPC, 2013).

All the veterinary professionals sampled in this study harbored CoPS species. These results substantiate that these professionals are very likely to be colonized by CoPS species, like are the pet owners (Hanselman *et al*., 2009; Frank *et al*., 2009; Morris *et al.,* 2010). However, the potential risk to veterinary professionals health still to be investigated.

Regarding the clinical environment, only a small percentage of objects harbored CoPS. However, a larger number of collected samples would certainly provide more information about these two *Staphylococcus* species present in the clinical environment. The presence of a MRSP with such a high antibiotic resistance pattern in the clinical environment can be worrisome in terms of public health and underlines the need of an exhaustive disinfection of clinical surfaces as well as good hand hygiene on the part of all veterinary professionals.

The combined analysis of isolates from small animals, veterinary professionals and clinical environment led us to conclude that there was a MSSA phenotype common to eight veterinary professionals, one clinical object (computer keyboard) and three animals (AMP^R P^R). A MSSP phenotype was common to one veterinary professional and four dogs (AMP^R P^R) and two MRSP isolates (one from a dog and one from the computer keyboard showed the same resistance pattern comprising simultaneous resistance against ampicillin, lomefloxacin, oxacillin, clindamycin, ciprofloxacin, amoxicillin, erythromycin, gentamicin, neomicin, sulfamethoxazole/trimethoprim, kanamycin, streptomycin and penicillin. These findings may be an indication of possible cross

contamination and dissemination of *S. aureus* and *S. pseudintermedius* clones among the three types of carriers analyzed in this study. Although the colonization mechanism remains unknown, a longitudinal study could provide additional information on how these contaminations might occur.

5. Conclusion

In this study, a phenotypic identification method, using CHROMagar Staph aureus, turned out to be very reliable in the identification of *S. aureus* and *S. pseudintermedius* isolated from animal, human and abiotic sources and, thus, can be very helpful in veterinarian clinical diagnostic practices. CoPS isolated herein showed diverse antimicrobial resistance patterns and several methicillin-resistant *Staphylococcus* species were found in the different sources sampled, underlining that dissemination of resistance clones is very likely to happen in the veterinary environment. Therefore, our results highlight the necessity of taking precautions in order to avoid the spread of multidrug-resistant strains, and in particular methicillin-resistant *Staphylococcus*, among animals and humans (owners and veterinary professionals).

Supplementary Data

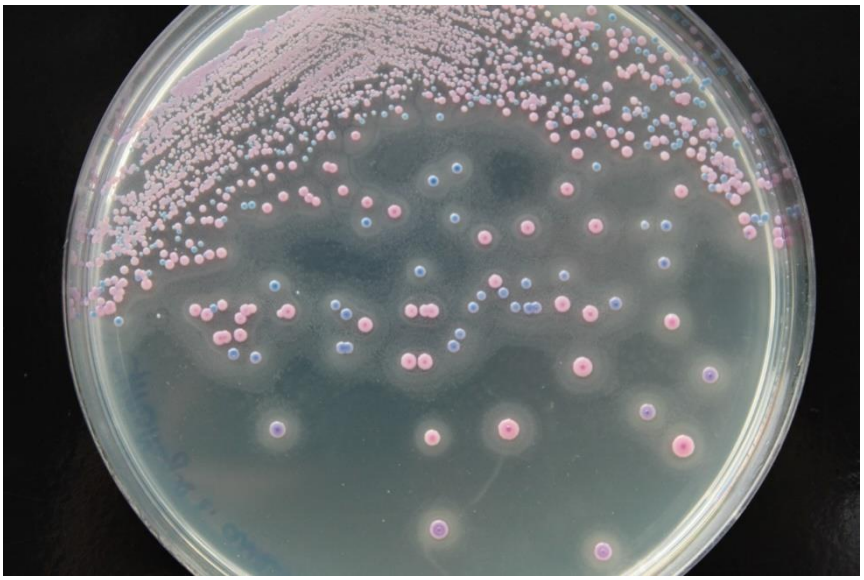

Figure I. Macroscopic image of CHROMagar Staph aureus medium with purple and blue colonies (*S. pseudintermedius*) and mauve and dark mauve colonies (*S. aureus*).

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Artigo em revista de circulação nacional com arbitragem científica

TRANSFERÊNCIA DE BACTÉRIAS RESISTENTES AOS ANTIMICROBIANOS ENTRE NICHOS ECOLÓGICOS INTERLIGADOS: HOMEM, ANIMAIS E AMBIENTE

Martins da Costa, P., Leite-Martins, L., Antunes, F., Simões, R.

Revista da Faculdade de Medicina de Lisboa, série III. 2010. 15 (5/6): 319-326.

Transferência de Bactérias Resistentes aos Antimicrobianos entre Nichos Ecológicos Interligados: Homem, animais e ambiente

PAULO MARTINS DA COSTA*,**, LILIANA LEITE-MARTINS*, FILIPA ANTUNES* e ROMEU SIMÕES*

RESUMO

A crescente prevalência de resistências aos antimicrobianos tem suscitado uma enorme apreensão. O uso desregrado de antimicrobianos durante as últimas décadas tem sido apontado como o principal impulsionador da emergência de microrganismos resistentes. No entanto, existe actualmente um maior enfoque científico na possibilidade de haver uma disseminação passiva de resistências em consequência das interdependências ecológicas existentes. Nesta perspectiva, o conjunto das resistências antimicrobianas existentes nas diversas populações animais constitui não somente um problema de saúde veterinária mas também um problema de saúde pública, em virtude de se poderem transferir directa ou indirectamente para o Homem.
Palavras-chave: antimicrobianos; resistências; Homem; animais; ambiente.

ABSTRACT

The increasing prevalence of antimicrobial resistance has raised serious concerns for human medicine. The use and misuse of antimicrobials has been claimed to be the driving force in the emergence of bacterial resistance during the past few decades, but there is also evidence for the epidemic spread of drug-resistant bacteria as a contributing factor. Thus, antimicrobial resistance in animals is an important veterinary health problem but is also a public health problem. Animal resistance pool may be transferred to other animals or humans either through direct contact, contamination of meat or, more indirectly, through environmental pathways. This ecological framework provides an essential perspective for formulating antimicrobial use policies precisely because it encompasses the root causes of these problems rather than merely their symptoms.
Key-words: antimicrobials; resistance; humans; animals; environment.

RFML 2010; Série III; 15 (5/6): 319-326

1. INTRODUÇÃO

A resistência aos antimicrobianos é um efeito com muitas causas e uma causa com muitos efeitos. Este fenómeno, assente na plasticidade genética das bactérias, emergiu em consequência da pressão selectiva resultante do uso de antibióticos em medicina humana, medicina veterinária, produção pecuária, aquacultura, agricultura e tecnologia alimentar;[1] exacerbou-se pela densidade selectiva resultante da sobre-prescrição de antibióticos e da ampliação das necessidades de prescrição em medicina humana consequente ao acréscimo dos procedimentos médicos invasivos e ao aumento do número de pacientes imunocomprometidos ou com doenças crónicas debilitantes;[2-4] globalizou-se pela mobilidade crescente de pessoas e mercadorias (alimentos) e pela inexistência de barreiras ambientais entres as diferentes comunidades vivas.[5,6]

Sempre que se usam antibióticos, as bactérias desenvolvem invariavelmente mecanismos de resistência através de uma alteração genética espontânea (mutação) ou pela aquisição de genes presentes noutras bactérias. Esta aquisição pode ocorrer por transdução (mediada por bacteriófagos), conjugação (possibilitando a transferência de plasmídeos ou transposões) ou transformação (aquisição de ácido desoxirribonucleico (ADN) livre resultante da lise bacteriana).[7] As resistências adquiridas pela transferência horizontal de genes caracterizam-se

* ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto.
** CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto.

pela propagação rápida e inter-específica, sobretudo num ecossistema poli-microbiano (*e.g.* intestino, mucosa respiratória e pele) e na presença de antibióticos.[4,8,9]

A coexistência de diversos genes de resistência no mesmo plasmídeo ou transposão implica a transferência acidental de todo o conjunto, mesmo quando a pressão selectiva antimicrobiana se dirige especificamente a um dos genes.[1,10] Este mecanismo de co-selecção, impossibilita *a priori* o estabelecimento de uma relação linear entre o uso específico de um antibiótico e a emergência de resistências a esse antibiótico.[7,8] O facto da célula receptora adquirir todas as competências genéticas e fenéticas existentes num determinado plasmídeo pode ter consequências ainda mais complexas, nomeadamente a transferência de factores de virulência na presença de um antibiótico, ou inversamente a transmissão acidental de genes de antibiorresistência pressionada pela presença de metais pesados ou desinfectantes[4,11] (Figura 1).

Apesar dos progressos tecnológicos e dos notáveis avanços da ciência no domínio da genética molecular assistiu-se, a partir da década de 70 do século XX, a um declínio muito significativo na descoberta de novos antibióticos.[12,13] Em sentido inverso, têm emergido a um ritmo muito preocupante, estirpes bacterianas multi-resistentes.[2] O insucesso terapêutico associado às resistências antimicrobianas aumenta globalmente a morbilidade e a mortalidade, com impactos notáveis na esfera individual, social e económica.[14] Num mundo sem antibióticos a probabilidade de morrer precocemente devido a uma infecção seria 40 % mais elevada.[15]

Para além destes efeitos clínicos directos e indirectos (*e.g.* necessidade de recorrer a antimicrobianos mais caros, tóxicos e difíceis de administrar), associam-se à resistência antimicrobiana consequências ecológicas e epidemiológicas igualmente gravosas. As primeiras consistem num aumento paradoxal do risco de aparecimento de doença clínica secundária à administração de um antibiótico. Este aumento no número de infecções (*excess cases*) é motivado pelo enriquecimento de microrganismos com resistências naturais ou adquiridas e, simultaneamente, pela disrupção do equilíbrio microflorístico decorrente da inibição da flora comensal sensível existente na pele e nas mucosas respiratória e digestiva.[9,10,16-18] Em 1962, Bohnhoff e Miller[19] tinham já demonstrado que a inibição parcial da flora entérica em ratos, através da administração prévia de estreptomicina, reduzia 100 000 vezes a dose infectante por *Salmonella* resistente à estreptomicina. Mais recentemente, Barza e Travers (2002)[16] estimaram que 13 a 66 % das salmoneloses humanas podem ser atribuídas à administração prévia de um antibiótico (*e.g.* para o tratamento de uma amigdalite) a pacientes portadores assintomáticos de uma estirpe de *Salmonella* resistente a esse antibiótico.

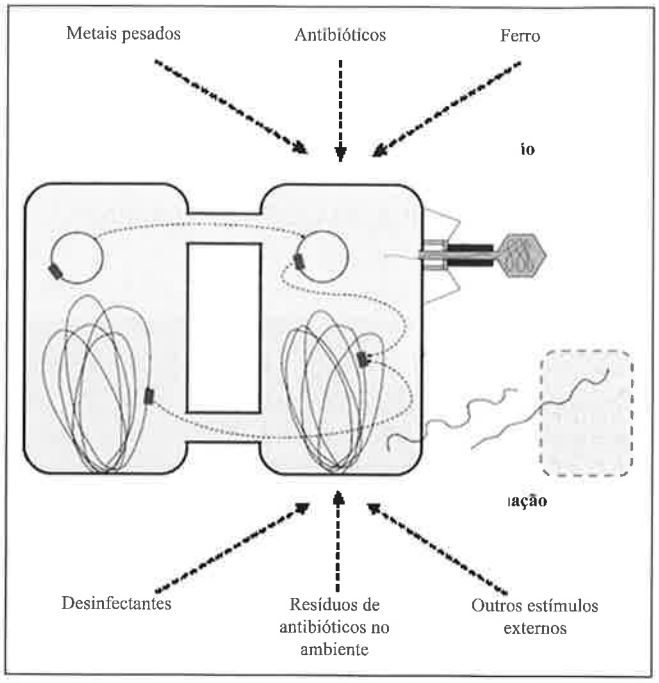

Figura 1. Aquisição, transferência e estabilização da antibiorresistência. As bactérias podem adquirir ADN exógeno contendo genes de resistência por um processo de transducção (mediado por bacteriófagos), transformação (aquisição de ADN livre resultante da lise microbiana) e conjugação, estabelecendo pontes citoplasmáticas através das quais transferem plasmídeos (círculos) e transposões (pequenos rectângulos cinzentos). Os transposões podem deslocar-se entre o cromossoma bacteriano e os plasmídeos. Após a selecção, diversos factores podem influenciar a dispersão e estabilização dos genes de resistência. Mutações compensatórias nestes genes perpetuam a sua persistência no genoma bacteriano mesmo na ausência de pressão selectiva. Adaptado de Barbosa e Levy (2000).[7]

Por sua vez, as consequências epidemiológicas resultam do facto de qualquer antibiótico provocar um enriquecimento massivo de estirpes resistentes, aumentando por um lado a probabilidade de contágio directo entre indivíduos inter-relacionados (*e.g.* pacientes internados e visitantes) e por outro a probabilidade de disseminação indirecta através das fontes ambientais contaminadas (*e.g.* água de recreio, consumo ou de rega).[14,16,20,21] Atendendo a que em cada população existe um reservatório de microrganismos resistentes (*e.g.* pneumococos resistentes à penicilina, enterococos resistentes à vancomicina, *Escherichia coli* multi-resistentes, *Staphylococcus aureus* resistentes à meticilina), os antimicrobianos serão sempre um importante acelerador da dispersão destes microrganismos, alargando

proporcionalmente as fronteiras temporais e geográficas da sua existência.[1,9] Este fluxo dinâmico, segundo a qual genes e vectores de resistência circulam ao longo de uma rede de hospedeiros medicados - bem patente nos hospitais e explorações pecuárias intensivas - favorece igualmente a estabilização destes elementos genéticos, ao ponto de se tornarem competitivos e persistentes mesmo na ausência do antibiótico respectivo,[10,14,22,23] contrariando uma expectativa inicial baseada nos resultados de trabalhos realizados nas décadas de 60 e 70 do século XX que reportavam uma forte tendência para as estirpes resistentes perderem espontaneamente, *in vitro*, os seus plasmídeos na ausência de pressão antibiótica.[24] Modelos matemáticos desenvolvidos por Austin et al. (1999)[25] demonstram que a escala temporal necessária para a emergência de resistência sob uma pressão antibiótica constante, é significativamente mais curta do que o período necessário para a sua regressão após suspensão do seu uso.

O acto médico de prescrição de antibióticos, assente numa visão excessivamente antropocêntrica dos microrganismos patogénicos, tende a ignorar a existência de um ciclo ecológico através do qual bactérias resistentes, seleccionadas pela administração de antibióticos em outros domínios, como a medicina veterinária, podem colonizar ou, pelo menos, transferir genes de resistência para bactérias patogénicas e comensais humanas.[2,17,26-29] Esta transferência pode ocorrer através do contacto directo do Homem com os animais ou por via alimentar, através do consumo de carne, peixe, ovos e leite.[30,31]

2. IMPACTO DO USO DE ANTIMICROBIANOS EM ANIMAIS

2.1. Animais de Companhia

Diversos relatórios têm enfatizado a necessidade de condicionar o uso de determinados antimicrobianos em cães e gatos (*e.g.* cefalosporinas e monobactâmicos) e, simultaneamente, promover medidas que mitiguem a dispersão de resistências antibióticas através da flora bacteriana de animais de companhia, seja no espaço público, seja no ambiente doméstico. Neste último, o contacto quotidiano potencia a transferência de flora microbiana, directamente através da pele ou do contacto com saliva e fezes, ou indirectamente, através das superfícies e objectos domésticos.[32-34]

Do universo de utentes da Clínica Veterinária do ICBAS/UP, uma família detentora de uma cadela medicada com diversos antibióticos, devido a uma infecção cutânea recidivante, disponibilizou-se para participar num estudo direccionado para a identificação dos factores de risco envolvidos na disseminação de bactérias resistentes, ou do respectivo material genético, entre cães e gatos e os seus proprietários. De um total de 124 isolados de *E. coli* obtidos a partir de amostras recolhidas na cadela, nos co-habitantes humanos e no ambiente doméstico, 24 estirpes exibiram um perfil de resistência bastante complexo. Os estudos de genética molecular (*PCR* e *PFGE*) demonstraram a partilha dos mesmos genes de resistência e a presença das mesmas estirpes nas fezes, urina e boca da cadela, nas fezes dos proprietários e em diversos pontos do ambiente doméstico, nomeadamente pavimento da habitação, taça da comida da cadela e puxador da porta do frigorífico (dados não publicados). O contacto directo entre os co-habitantes e o toque de superfícies e objectos domésticos foram, muito provavelmente, responsáveis pela disseminação das estirpes. Conforme demonstrado em estudos anteriores revistos por Tollefson e Karp (2004),[29] no momento em que uma bactéria portadora de resistências aos antimicrobianos se transfere para um novo hospedeiro, a sua capacidade para infectar ou colonizar pode ser muito variável. Ainda assim, mesmo quando a permanência é apenas transitória, a bactéria pode ter a oportunidade de transferir as competências de que é portadora ou receber as competências de outras bactérias, comensais ou patogénicas.[17,30]

2.2. Espécies Pecuárias

Ao longo dos últimos 40 anos, a evolução demográfica, o desenvolvimento tecnológico, a alteração dos hábitos alimentares e a globalização dos mercados causaram profundas alterações nos sistemas de produção animal e vegetal, predominando actualmente o regime intensivo em sistema fechado.[27,28] A actual performance produtiva das espécies pecuárias (produtoras de carne, ovos e leite) depende da ausência de estados mórbidos para a sua expressão optimizada sendo os antimicrobianos uma ferramenta essencial para a satisfação desta necessidade.[35,36] No entanto, o uso sistemático de antimicrobianos nos efectivos animais implica, inevitavelmente, a emergência de resistências.[28,29,36,37]

A possibilidade de ocorrer um insucesso terapêutico em medicina humana devido a resistências seleccionadas pelo uso de antimicrobianos em espécies pecuárias constitui uma importante ameaça à saúde pública, sendo actualmente uma matéria de grande enfoque científico e social.[29,31] As resistências seleccionadas no domínio pecuário repercutem-se negativamente na saúde humana, pela emergência de bactérias zoonóticas multi-resistentes (*e.g. Salmonella* spp., *Campylobacter jejuni, Listeria monocytogenes*, *Yersinia enterocolitica*) e pela possibili-

dade dos determinantes genéticos de resistência seleccionados na flora comensal dos animais (*e.g. E. coli*, *Staphylococcus aureus* e *Enterococcus* spp.) se transferirem para organismos patogénicos humanos.[28,30] O contacto directo do Homem com os animais, a exposição a fontes ambientais (águas, solos, vegetais e insectos) contaminadas com as excreções, estrumes, águas residuais e cadáveres das explorações pecuárias, mas sobretudo a manipulação e o consumo de carnes, ovos e alguns produtos lácteos, constituem o suporte físico de transferência destas bactérias para o microbiota humano.[1,18]

Trabalhos efectuados em condições de campo por Martins da Costa et al. (2008b, 2009a, 2009b, 2010)[36,38-40] com o objectivo de avaliar o impacto selectivo em *E. coli* e *Enterococcus* spp. decorrente do uso preventivo de antimicrobianos durante o período de cria de frangos, permitiram concluir que a pressão selectiva exercida por estas substâncias se manifesta de uma forma impressiva, cooperativa e cumulativa (Figura 2).

Recorde-se que o paradigma de utilização dos antimicrobianos em explorações intensivas tem indisfarçáveis semelhanças com o seu uso a nível hospitalar; em ambos os biomas usam-se grandes quantidades de antimicrobianos, pertencentes a famílias químicas muito diversas, havendo um microbismo residente sujeito a uma pressão selectiva sucessiva.[39,41] Sendo evidente que nas explorações pecuárias não se usam antimicrobianos de última geração, esta diferença é contra-balançada pelo facto dos antimicrobianos serem (i) maioritariamente usados em concentrações sub-inibitórias, bastante mais selectivas comparativamente às terapêuticas[17,42] e (ii) maioritariamente administrados por via oral.[37] No biótopo intestinal, a intensa divisão celular e a taxa de erro associada à replicação do ADN proporcionam uma grande constelação de mutações genéticas que codificam alterações estruturais ou funcionais capazes de conferir a uma célula microbiana a capacidade para resistir a um determinado agente antimicrobiano.[7,10,27] A administração desse agente determinará uma rápida disseminação destes novos clones resistentes. Por outro lado, a grande diversidade microflorística e o contacto estreito entre as células microbianas no ambiente intestinal proporcionam, respectivamente, uma grande variedade de genes de resistência e a oportunidade para a sua rápida transferência entre células microbianas através de elementos genéticos móveis.[31] Esta transmissão ocorre preferencialmente entre bactérias com grande proximidade filogenética, mas pode também verificar-se entre espécies e géneros diferentes.[10]

Os trabalhos anteriormente citados[36,38-40] permitiram verificar que, no caso específico dos enterococos, a

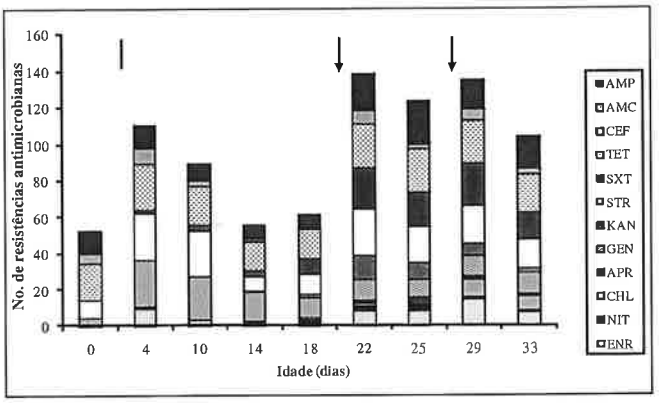

Figura 2. Evolução do número total de resistências antimicrobianas em *E. coli* isoladas em 9 amostras de fezes recolhidas no grupo Medicado ao longo do ciclo de crescimento. De cada amostra recuperaram-se, aleatoriamente, 26 isolados. As aves foram medicadas com a associação lincomicina/espectinomicina entre o 1.º e 3.º dias, a associação sulfadiazina/trimetoprime entre o 19.º e o 21.º dias, e tilosina entre o 26.º e o 28.º dia de idade. As setas indicam o momento em que os antimicrobianos foram administrados. Foram pesquisadas resistências relativamente a amoxicilina/ácido clavulânico (AMC), ampicilina (AMP), apramicina (APR), cefalotina (CEF), cloranfenicol (CHL), enrofloxacina (ENR), gentamicina (GEN), canamicina (KAN), nitrofurantoína (NIT), estreptomicina (STR), tetraciclina (TET) and sulfametoxazole/trimetoprime (SXT).[38]

presença abundante destas bactérias nos alimentos compostos (rações) fornecidos às aves provocava um "efeito de diluição" dos fenómenos desencadeados pela pressão selectiva antibiótica sobre os enterococos "nativos". Esta observação demonstra que a prevalência de fenótipos resistentes transcende, relativamente a este género microbiano, a mera interacção de um antimicrobiano específico com uma determinada população microbiana, num espaço e tempo definidos. Dito de outra forma, relativamente a este Género microbiano a frequência global de resistência era mais influenciada pelo fluxo passivo de enterococos veiculados nos alimentos (500 000 UFC/kg, cujo perfil de resistência tinha sido modulado noutros habitats) do que pela administração periódica de antimicrobianos às aves.[35]

3. DISPERSÃO AMBIENTAL DE RESISTÊNCIAS A PARTIR DE ESTAÇÕES DE TRATAMENTO DE ÁGUAS RESIDUAIS (ETAR)

As resistências seleccionadas na abundante flora entérica pela administração de antimicrobianos a humanos ou a animais dispersam-se no ambiente através dos efluentes. Trabalhos realizados entre 2002 e 2006[43-46] com o objectivo de determinar a prevalência de resistência em *E. coli* e *Enterococcus* spp. isolados a partir de amostras de efluentes e lamas resultantes do

tratamento de esgotos urbanos e do tratamento de águas residuais provenientes do abate de frangos de carne permitiram verificar que os processos de depuração destas águas têm uma fraquíssima capacidade de conter a dispersão de fenótipos multi-resistentes nos ambientes terrestre e aquático, verificando-se, na quase generalidade das ETAR, que a redução do número de *E. coli* e *Enterococcus* spp. no efluente final resultava não propriamente da eliminação destas bactérias, mas sim de sua transferência para as lamas produzidas. A esmagadora maioria das lamas produzidas é utilizada como fertilizante em jardins públicos e campos agrícolas. Desta forma o Homem, mas também os animais, ficam permanentemente expostos à colonização por bactérias, cujos perfis de resistência foram determinados pela pressão selectiva exercida por antibióticos remotamente administrados. Esta observação demonstra que a selecção de fenótipos resistentes transcende a mera interacção de um antibiótico específico com uma determinada população microbiana, num espaço e tempo definidos.[5,30]

Na análise qualitativa, verificou-se que o nível de urbanização e o número e a dimensão das unidades de saúde influíram na prevalência de resistências registada nas 14 ETAR urbanas testadas. Verificou-se também que a prevalência de resistências nos isolados avícolas era sensivelmente o dobro da verificada nos isolados humanos. Tendo em conta que, na época, a população portuguesa ocupava o quarto lugar em consumo de antibióticos dentro da União Europeia,[47] e que as ETAR urbanas recebem também efluentes de hospitais e centros de saúde, os resultados confirmam o uso superlativo de antibióticos em produção avícola.

4. PRESENÇA DE RESISTÊNCIAS EM ANIMAIS NÃO MEDICADOS

Mais recentemente, tem vindo a ser reportada a presença de bactérias multi-resistentes em populações de aves (e.*g.* gaivotas, aves de rapina, lobos, lontras) e mamíferos selvagens sem exposição aparente aos antimicrobianos.[48-51] Estas descobertas sugerem que a resistência, uma vez desenvolvida, não se confina aos limites do nicho ecológico em que primariamente emergiu.

4.1. Gaivotas

Na orla costeira do Porto, e mesmo no centro urbano desta cidade, existe uma grande população de gaivotas (*Larus fuscus, L. cachinnans* e *L. ridibundus*). Quinzenalmente, entre Dezembro de 2007 e Abril de 2008, recolheram-se amostras de fezes de gaivotas nas praias de Matosinhos e de Leça da Palmeira, com o objectivo de quantificar a ocorrência de *E. coli* produtoras de β-lactamases de espectro alargado (ESBL) isoladas a partir das fezes dessas gaivotas. De um total de 139 estirpes multi-resistentes, 45 revelaram ser ESBL. Os genes responsáveis por este fenótipo foram identificados por PCR e, posteriormente, sequenciados tendo-se obtido as seguintes frequências: 18 % CTX-M 1 (n=8), 9% CTX--M 9 (n=4), 39 % CTX-M 15 (n=17) e 34 % CTX-M 32 (n=15). Foram também determinados os filogrupos (A, B1, B2 e D) por PCR, verificando-se que 42 % das estirpes de *E. coli* pertenciam aos grupos potencialmente mais virulentos (B2 e D), sendo assinalável que todos os isolados do filogrupo B2 produziam CTX-M 15 ou CTX-M 32, ou seja aquelas que conferem um maior grau de resistência às cefalosporinas.[51]

A elevada frequência do determinante CTX-M 15, só muito recentemente identificado em estirpes de *E. coli* isoladas em humanos tratados com cefalosporinas,[52] sugere a rápida disseminação destes genes às estirpes isoladas nas gaivotas. Desta forma, esta numerosa população de aves passa a constituir um importante reservatório de estirpes ESBL, potenciando a sua "devolução" à população humana. Esta transmissão é favorecida pelo contacto estreito entre estas aves e a população humana (*e.g.* quando executa actividades de recreio nas praias). Acresce que sendo a *Larus fuscus* uma ave migratória, o potencial de disseminação destas estirpes multi-resistentes estende-se por toda a orla costeira percorrida, ou seja até à Escandinávia.[51]

A presença destes fenótipos multi-resistentes em gaivotas demonstra que os efeitos selectivos dos antimicrobianos não se extinguem nos indivíduos medicados. Na verdade, os esgotos em que estas aves procuram alimento garantem as condições ideais para a emergência de resistências antibióticas, nomeadamente i) um bioma polimicrobiano que abone uma grande diversidade genética e ii) uma concentração antibiótica sub-inibitória (*i.e.* insuficiente para inactivar as bactérias mas suficientemente elevada para as pressionar) devido à eliminação, em natureza, destas substâncias nas fezes e urina de indivíduos sob antibioterapia.[53-55]

Este processo, clinicamente invisível por ocorrer num ponto distante do acto de prescrição, implica que quanto maior for a quantidade de antimicrobianos prescrita, maior será a sua dispersão na biosfera, aumentando a probabilidade de num determinado bioma (*e.g.* rio, lago, orla costeira) uma grande população microbiana contactar com a concentração selectiva ideal desses antimicrobianos.[5]

4.2. Aves Selvagens

Com o objectivo determinar a prevalência de estirpes *E. coli* resistentes aos antimicrobianos em aves selvagens (*e.g.* Águia-de-asa-redonda, Coruja-das-torres, Milhafre-preto, Grou) iniciou-se em Março de 2008 a recolha de zaragatoas cloacais de aves que ingressaram no Centro de Recuperação do Parque Nacional da Peneda-Gerês e no Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens do Parque Natural da Serra da Estrela. A partir de 175 aves amostradas, obtiveram-se 139 estirpes multi-resistentes, *i.e.* evidenciado resistências a mais de três antimicrobianos, e 53 estirpes produtoras de β-lactamases de espectro alargado.[50] A detecção destes fenótipos em aves silvestres provenientes de habitats pouco ou nada humanizados revela-se, à partida, um achado muito surpreendente em virtude destas estirpes terem vindo a ser detectadas sobretudo em pacientes humanos hospitalizados.[52] Duas hipóteses podem justificar a presença destas estirpes em aves de vida selvagem: i) a ingestão de quantidades residuais de antimicrobianos em concentrações suficientemente elevadas para pressionarem a emergência de resistências na flora entérica destas aves. Estes antimicrobianos podem estar presentes no habitat (libertados a partir dos efluentes urbanos e/ou pecuários) ou, no caso das aves silvestres carnívoras ou necrófagas, a contaminação pode ocorrer através da ingestão de presas sujeitas a tratamentos com antimicrobianos; ii) a partilha de elementos genéticos transferíveis que codifiquem resistências entre bactérias provenientes de fontes ambientais (*e.g.* água ou alimento) e a flora entérica nativa das aves selvagens.

4.3. Lobos

Durante o ano de 2009, processaram-se 26 amostras fecais recolhidas em lobo-ibérico (*Canis lupus signatus*) ao abrigo dos projectos de estudo e conservação da espécie coordenados pelo Centro de Investigação em Biodiversidade e Recursos Genéticos da Universidade do Porto (CIBIO-UP). O lobo-ibérico é considerado um dos mamíferos mais ameaçados no nosso país, principalmente devido à perseguição humana (envenenamento, caça furtiva, atropelamento), à escassez de presas adequadas e à fragmentação do habitat natural devido à expansão da área humanizada com terrenos agrícolas, barragens e auto-estradas. A população lupina em território nacional (estimada em 200 a 400 espécimes) está dividida em duas sub-populações: a mais numerosa (entre 45 e 55 alcateias) encontra-se a norte do rio Douro, e tem contacto com a população espanhola; uma outra, a sul do mesmo rio e aparentemente isolada da restante população ibérica, não ultrapassa as 10 alcateias.[56] No conjunto das amostras recolhidas detectou-se um número muito significativo de estirpes, sem qualquer relação clonal, portadoras de β-lactamases de espectro alargado, confirmando uma vez mais a dispersão ambiental destes fenótipos na ausência de uma pressão selectiva específica. A ingestão de presas sujeitas a tratamentos com antimicrobianos (*e.g.* espécies pecuárias) afigura-se como a via mais provável de contaminação. Os ungulados silvestres - as presas naturais do lobo - apresentam também um declínio populacional, sobretudo devido ao avanço das áreas humanizadas e à criação de gado em regime extensivo. Desta forma, também os hábitos alimentares das populações lupinas sofreram alterações, estando documentado o recurso a ungulados domésticos, sobretudo os criados em regime extensivo, e a cadáveres provenientes de explorações intensivas.[57]

O facto das populações lupinas passarem a constituir reservatório de estirpes multi-resistentes representa um problema de saúde ambiental e, simultaneamente, uma ameaça acrescida à conservação da espécie, em virtude destas bactérias multi-resistentes albergarem diversos factores de virulência que lhes conferem capacidade acrescida para colonizarem e causarem patologia nos hospedeiros, particularmente quando estes se encontram imuno-deprimidos em consequência de infestações parasitárias, infecções víricas (*e.g.* parvovirose) ou carências nutritivas.

5. A BIODIVERSIDADE E A RESISTÊNCIA AOS ANTIMICROBIANOS

A biodiversidade é um elemento fundamental para o equilíbrio e regeneração dos ecossistemas. Desde há muito que se reconhece na biodiversidade um elemento essencial para a interrupção dos ciclos de propagação de agentes infecto-contagiosos. Curiosamente, este efeito é extensível às próprias resistências antibióticas, envolvendo aliados improváveis na redução do reservatório ambiental de bactérias resistentes.

Os bivalves filtram e acumulam uma elevada quantidade de microrganismos presentes na água. A relação biótica que estes organismos filtradores desenvolvem com as diferentes espécies microbianas presentes no seu habitat tem vindo a ser estudada. No caso particular da *Anodonta cygnea* - bivalve de água doce ameaçado de extinção, presente em três lagoas do centro do país[58] - verificou-se em espécimes recolhidos na lagoa de Mira que na sua hemolinfa e fluido extrapaleal se poderiam

contabilizar entre $1,5\times10^2$ e $6,5\times10^2$ UFC ml^{-1} células microbianas cultiváveis, predominando *Vibrio metschnikovii* e *Aeromonas sobria*. Todavia, durante o trabalho não se detectou a presença de *E. coli* e *Enterococcus* spp., apesar das análises efectuadas à água e sedimento envolventes revelarem uma presença muito abundante destes microrganismos.[59] Na tentativa de responder a esta observação, verificou-se que as células imunitárias circulantes na hemolinfa destes bivalves (granulócitos) tinham capacidade para fagocitar activa e especificamente estas espécies microbianas, sendo esta capacidade mais intensa perante estirpes virulentas e com grande capacidade de resistência aos antimicrobianos (Figura 3).

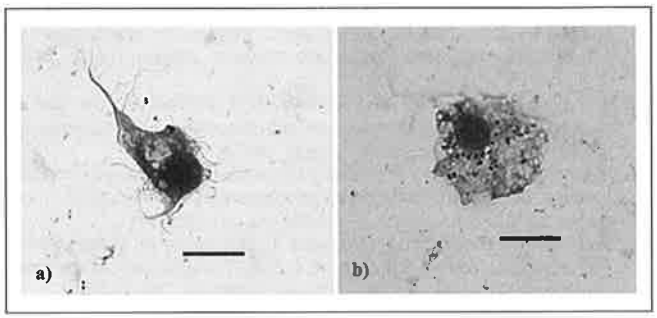

Figura 3. Granulócito de *Anodonta cygnea* evidenciando longas projecções (pseudópodes) (a) e fagocitose de bacilos Gram-negativo (*E. coli*) por um granulócito em apoptose (b). A barra de escala equivale a 10 µm.[59]

Para além da relevância biológica destas observações, procura-se esclarecer o eventual interesse ecológico e médico das mesmas. Numa perspectiva ecológica, a capacidade destes bivalves para filtrarem e eliminarem bactérias perigosas nos ecossistemas aquáticos pode ter um grande interesse, em virtude acelerar o declínio destas bactérias, reduzindo a probabilidade delas serem reintroduzidas em hospedeiros humanos ou animais. Na vertente médica, será extremamente relevante identificar os mecanismos imunitários subjacentes ao reconhecimento, captura e destruição de estirpes de *E. coli* e *Enterococcus* spp. virulentas e com elevado número de resistências antimicrobianas.

6. CONCLUSÕES

Os antimicrobianos são uma inestimável ferramenta para salvar vidas. O uso veterinário destas substâncias em produção pecuária e o uso médico como elemento para a promoção do bem-estar, acompanhando a evolução recente do conceito de saúde, criaram uma pressão selectiva muito poderosa (à escala mundial, estima-se que a quantidade total de antibióticos usados oscile entre 100 a 200 mil toneladas por ano) que se perpetua, no espaço e no tempo, muito para além do acto de prescrição.[5]

Assim, mesmo que por hipótese académica pudéssemos suprimir o uso de antimicrobianos, a actual dispersão de bactérias multi-resistentes na biosfera implica um nível assinalável de exposição da população humana a estas bactérias através dos alimentos e da água.[4,18,60]

Somente uma abordagem holística, capaz de identificar as inúmeras possibilidades de transferência destes microrganismos resistentes entre nichos ecológicos interligados, possibilita a compreensão plena dos efeitos selectivos dos antibióticos. Esta proposta de avaliação interdisciplinar - erigida sobre o lema "One Health" - pretende encorajar a colaboração entre médicos, veterinários e ambientalistas com o intuito de compreenderem este, e outros problemas, encontrando soluções multidisciplinares para proteger e promover a saúde de todas as espécies.

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Comunicação Oral

PREVALÊNCIA DA RESISTÊNCIA AOS ANTIMICROBIANOS EM *ESCHERICHIA COLI* E *ENTEROCOCCUS* SPP. ISOLADOS EM CÃES E GATOS E ESTUDO DOS RESPETIVOS FATORES DE RISCO

Liliana Leite-Martins

VIII Congresso OMV. Lisboa, Portugal. 30 de Novembro a 01 de Dezembro de 2013.

Prevalência da resistência aos antimicrobianos em *Escherichia coli* e *Enterococcus* spp. isolados em cães e gatos, e estudo dos respetivos fatores de risco

L. R. Leite-Martins[1], C. Frias[1], J. Santos[1], J. Ribeiro[1], V. Santos[1], C. Abreu[1], J. Niza-Ribeiro[2], A. J. de Matos[1], P. Martins da Costa[3]

[1] UPVET, Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

[2] Departamento de Estudos das Populações, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

[3] Departamento de Microbiologia e Tecnologia Alimentar, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

Abstract

Este estudo teve como objectivos: i) a determinação da prevalência da resistência aos antimicrobianos em *E. coli* e enterococos fecais isolados de cães (n=78) e gatos (n=22) atendidos no Hospital Veterinário da Universidade do Porto (UPVET); e ii) a análise da correlação estatística dos factores de risco que, potencialmente, a influenciam.

As amostras foram recolhidas de Setembro de 2009 a Maio de 2012. Após explicação dos objectivos do estudo, solicitou-se a cada proprietário participante o preenchimento de um questionário. A amostragem foi realizada através de zaragatoa rectal. O isolamento de *E. coli* (n = 398) e *Esterococcus* spp. (n = 315) realizou-se mediante sementeira em TBX agar e Slanetz & Bartley, respectivamente. A susceptibilidade aos antimicrobianos foi determinada através da técnica de difusão em agar. Para o processamento estatístico utilizou-se um modelo de análise multivariada multinível (GLMM), atendendo a que a cada animal correspondiam mais do que um isolado.

Os resultados destacaram-se pelas elevadas taxas de resistência de *E. coli*. O tratamento prévio com quinolonas e a prática de coprofagia foram os factores de risco mais significativamente associados à resistência a ciprofloxacina, cefalotina, cefoxitim, ceftazidima, cefotaxime, gentamicina, estreptomicina e trimetroprim-sulfametoxazol.

A co-selecção de resistências antimicrobianas fomentada pelo uso de quinolonas teve, provavelmente, um papel preponderante na elevada frequência e diversidade de resistências antimicrobianas. No caso particular da coprofagia, os efeitos podem transcender a “ingestão” de estirpes fecais presentes em outros animais, em virtude de quantidades subinibitórias de antimicrobianos também poderem estar presentes nas fezes. Estes resultados ilustram os efeitos selectivos resultantes da administração de antimicrobianos, poderão auxiliar a classe médico-veterinária a orientar a sua prescrição em função de eventuais impactos para a saúde pública e, não menos importante, estimulam os proprietários a adoptarem medidas de maneio e higiene mais seguras.

Comunicação Oral

O CICLO AMBIENTAL DAS RESISTÊNCIAS ANTIMICROBIANAS (*ENVIRONMENTAL DISSEMINATION OF DRUG-RESISTANT BACTERIA BETWEEN INTERMINGLED ECOLOGICAL NICHES*)

Martins da Costa, P., Simões, R., Martins, L., Matos, A.J.

V Congresso de Ciências Veterinárias 2011. Sociedade Portuguesa de Ciências Veterinárias. Santarém, Portugal. 14 de Outubro de 2011. (Pp.57).

Alimentos – Segurança Sanitária

Comunicação por convite

O ciclo ambiental das resistências antimicrobianas

Paulo Martins da Costa[1,2], Romeu Simões[1,2], Liliana Martins[1,3] e Augusto J.F. Matos[1,3]

1 ICBAS, Universidade do Porto, Portugal
2 CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Portugal
3 UMIB - Centro Multidisciplinar para a Investigação Biomédica, Universidade do Porto, Portugal

A resistência aos antimicrobianos é um efeito com muitas causas e uma causa com muitos efeitos. Este fenómeno, assente na plasticidade genética das bactérias, emergiu em consequência da pressão selectiva resultante do uso de antibióticos em medicina humana, medicina veterinária, produção pecuária, aquacultura, agricultura e tecnologia alimentar; exacerbou-se pela densidade selectiva resultante da sobre-prescrição de antibióticos e da ampliação das necessidades de prescrição em medicina humana consequente ao acréscimo dos procedimentos médicos invasivos e ao aumento do número de pacientes imunocomprometidos ou com doenças crónicas debilitantes; globalizou-se pela mobilidade crescente de pessoas e mercadorias (alimentos) e pela inexistência de barreiras ambientais entres as diferentes comunidades vivas. Somente uma abordagem holística, capaz de identificar as inúmeras possibilidades de transferência destes microrganismos resistentes entre nichos ecológicos interligados, possibilita a compreensão plena dos efeitos selectivos dos antibióticos. Esta proposta de avaliação interdisciplinar - erigida sobre o lema "One Health" - pretende encorajar a colaboração entre médicos, veterinários e ambientalistas com o intuito de compreenderem este, e outros problemas, encontrando soluções multidisciplinares para proteger e promover a saúde de todas as espécies.

[Environmental dissemination of drug-resistant bacteria between intermingled ecological niches]

Antimicrobial resistance is an effect with many causes and a cause with many effects. This phenomenon, based on genetic plasticity of bacteria, has emerged as a consequence of the selective pressure exerted by the antimicrobial usage in human medicine, veterinary medicine, animal production, fish production, agriculture and food technology. Antimicrobial resistance has exacerbated due to antibiotics over-prescription and increased use in human medicine, as a consequence of the growing number of invasive medical procedures and the enormous increase in the number of immunocompromised individuals and patients with chronic debilitating diseases. The increasing mobility of people and food products, as well as an absence of environmental barriers between different living communities, raise the risk of the spread of resistance world-wide. Antimicrobials are essential to save lives. Because resistance is becoming increasingly widespread without any plausible relationship with the use of antimicrobials, it is necessary to seriously consider other strategies in order to prevent the emergence and dissemination of antimicrobial resistant bacteria. These strategies require a more holistic and forward-looking approach that take the complex interconnections among species into full account, recognizing the important link between human and animal health in accordance with the Manhattan principles on "One World, One Health".

Comunicação Poster

ESTUDO DA PARTILHA DE CLONES BACTERIANOS ENTRE ANIMAIS DE COMPANHIA, COABITANTES HUMANOS E SUPERFÍCIES DOMÉSTICAS

Meireles, D.M., Martins, L.R., Bessa, L.J., Mendes, Â.J., Cunha, S.A., Matos, A., da Costa, P.M.

VI Congresso da Sociedade Portuguesa de Ciências Veterinárias: Praxis e futuro, Oeiras, Portugal, 3-5 de Abril de 2014. (Pp.127).

Estudo da partilha de clones bacterianos entre animais de companhia, coabitantes humanos e superfícies domésticas

Meireles, DM[1]; Martins, LR[1]; Bessa, LJ[2]; Mendes, ÂJ[1]; Cunha, SA[1]; Matos, A[1]; da Costa, PM[1,2].

[1] Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto.
[2] Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto

A pressão seletiva originada pelo uso de antimicrobianos na medicina humana e veterinária tem contribuído para a emergência de estirpes bacterianas multirresistentes. Porque os animais e os seus proprietários partilham o mesmo espaço habitacional, apresentando comportamentos de contacto próximo, existe uma hipótese elevada de transferência microbiana inter-espécie. Ante esta possibilidade é importante escrutinar o papel dos animais de companhia enquanto reservatórios assim como a sua envolvência na disseminação de estirpes bacterianas multirresistentes. Importa também, investigar o papel das superfícies e objetos domésticos, como potenciadores deste fenómeno. Assim, com este trabalho pretendeu-se inferir sobre a partilha de clones de *Escherichia coli* e *Enterococcus* spp. com elevadas resistências, em agregados familiares (humanos e seus animais de companhia) avaliando também a sua possível disseminação no ambiente doméstico.

Previamente, em animais que apresentavam historial de várias terapias antimicrobianas, consultados no Hospital Veterinário do ICBAS – UPVET, foram recolhidas zaragatoas de fezes, mucosa oral, pelo, e em alguns casos, dos seus proprietários e ambiente doméstico. O processamento das zaragatoas permitiu o isolamento de estirpes que foram submetidas a testes de suscetibilidade antimicrobiana e seleção de isolados com perfis de resistência similares. A técnica de multiplex PCR foi utilizada para caracterização de filogrupos (*Escherichia coli*) e identificação de espécie (*Enterococcus* spp.). A avaliação da proximidade clonal entre isolados foi efetuada por genótipagem (ERIC PCR e PFGE).

Nos "agregados familiares" estudados foi observada uma partilha frequente de clones de *Escherichia coli* e *Enterococcus faecalis* com múltiplas resistências, isolados em fezes, mucosa oral e pelo de cães e gatos e fezes e mãos dos respetivos proprietários, evidenciando-se assim uma possível transferência entre coabitantes, que pode ocorrer em ambos os sentidos. Ficou também comprovado com percentagens elevadas de similaridade genotípica que essa disseminação ocorre para o ambiente doméstico, envolvendo objetos dos animais e de uso comum. Os resultados obtidos reforçam a necessidade de um uso prudente dos antimicrobianos, pois elevados padrões de resistências terão um impacto na qualidade de vida dos animais e também na saúde humana. Adicionalmente importa sensibilizar os proprietários para a necessidade de uma maior vigilância relativamente às formas de interação com os animais, bem como para a adoção de medidas higiénicas cautelares após essa mesma interação.

Spread of multidrug-resistant bacterial strains between humans, pets, and household environment

Meireles, DM[1]; Martins, LR[1]; Bessa, LJ[2]; Mendes, ÂJ[1]; Cunha, SA[1]; Matos, A[1]; da Costa, PM[1,2].

[1] Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto.
[2] Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto

The selective pressure caused by the excessive use of antimicrobials, both in human and veterinary medicine, has triggered the emergence of multidrug-resistant bacterial strains. Since animals and their owners share the same living space, with close contact behaviors, there is a chance of inter-species microbial transfer. Taking into account this hypothesis, it is important to scrutinize the role of companion animals as a reservoir of bacterial strains, as well as their involvement in the dissemination of multidrug-resistant bacteria. It is also important to investigate the role of surfaces and objects shared by both animals and humans, as potential enhancers of this phenomenon. Hence, with this work, it was sought to infer about the sharing of *Escherichia coli* and *Enterococcus* spp. clones in households (humans and their pets) as well as observe their dissemination across the domestic environment.

Previously, in companion animals that had been prescribed with various antimicrobial therapies and that had appointments in the Veterinary Hospital of ICBAS-UP - UPVET, swabs from their feces, oral mucosa and hair were collected, and in some cases from their owners, as well as from the domestic environment. Swabs were processed and antimicrobial susceptibility tests were performed which allowed the selection of isolates with similar resistance phenotypes. Multiplex-PCR techniques were used to characterize phylogenetic groups (*Escherichia coli*), and to species identification (*Enterococcus* spp.). Genotyping techniques - ERIC PCR, PFGE - were used to study the clonal proximity between isolates.

In the studied households, it was observed the sharing of *Escherichia coli* and *Enterococcus faecalis* clones with multiple resistances, among cats and dogs' feces and oral mucosa, and their respective owners hands and feces, being evident that there was a possible direct transmission between cohabitants. Such transference can occur in both directions. It has also been demonstrated with large rates of similarity in the genotypic profile that this dissemination also occurs in the home environment, with transference to common use and animal objects. Regarding these results, it is easily noticeable that it is necessary to use antibiotics cautiously since high resistance levels will have an impact on pet's quality of life, but also on human health. Additionally, it's important to make the owners aware of the need of greater vigilance on how they interact with the animals, as well as for the adoption of precautionary hygienic measures after that interaction.

VI CONGRESSO
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ESTUDO DA PARTILHA DE CLONES BACTERIANOS ENTRE ANIMAIS DE COMPANHIA, COABITANTES HUMANOS E SUPERFÍCIES DOMÉSTICAS

Meireles, DM[1]; Martins, LR[1]; Bessa, LJ[2]; Mendes, ÂJ[1]; Cunha, SA[1]; Matos, A[1]; da Costa, PM[1,2]

Laboratório de Microbiologia e Tecnologia Alimentar
[1] Instituto de Ciências Biomédicas Abel Salazar (ICBAS) - Universidade do Porto, Portugal
[2] Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Portugal

Introdução

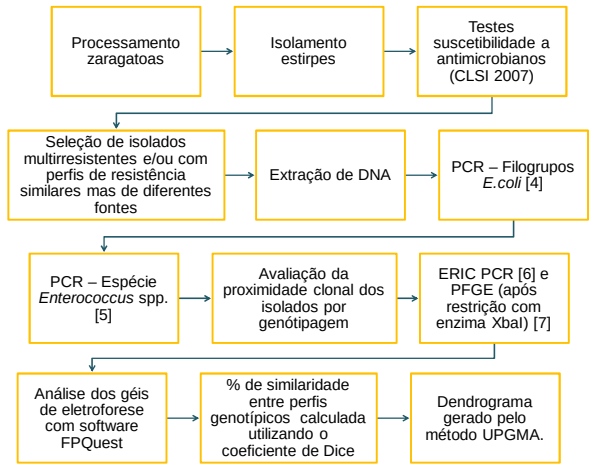
Avanços na medicina veterinária e uma maior sensibilidade por parte da população em relação à saúde e bem-estar dos animais de companhia estão associados com o aumento da população geriátrica, que necessita de múltiplas terapias antimicrobianas. Este facto leva à emergência de multirresistências tanto nas bactérias patogénicas como na flora comensal endógena. Atualmente, os animais de companhia e os seus proprietários partilham o mesmo espaço habitacional, apresentando comportamentos de contacto próximo, devido à corrente perceção destes animais como membros da família [1][2], pelo que existe uma hipótese elevada de transferência microbiana entre estes coabitantes. Ante esta possibilidade é importante escrutinar o papel dos animais de companhia enquanto reservatórios assim como o seu papel na disseminação de estirpes bacterianas multirresistentes. Importa também, investigar o papel das superfícies e objetos domésticos, como potenciadores deste fenómeno. Existem alguns estudos longitudinais envolvendo agregados que verificaram a transmissão de clones de *Escherichia coli* entre familiares e animais de estimação. Dentro de um agregado familiar foi verificado que o clone responsável pela ITU da mãe era partilhada por vários membros, incluindo o cão [3].

Objetivos

Neste trabalho pretendemos inferir sobre a partilha de clones de *E. coli* e *Enterococcus* spp. com elevadas resistências, entre os proprietários e os seus animais de companhia, avaliando também a sua possível disseminação no ambiente doméstico.

Métodos

Os objetivos do estudo foram explicados aos proprietários e uma autorização formal foi assinada**.** Em animais que apresentavam historial clínico de várias terapias antimicrobianas, consultados no Hospital Veterinário do ICBAS – UPVET (Porto, Portugal), foram recolhidas zaragatoas de fezes, mucosa oral e pelo. Quando os proprietários aceitaram colaborar na fase seguinte do estudo, foram pedidas zaragatoas das suas mãos e fezes, assim como colheitas noutros animais coabitantes e de superfícies/objetos de suas casas. Todos os participantes preencheram um questionário para recolha de um conjunto de informações pessoais e médicas.



Para interpretação da proximidade clonal dos isolados foi utilizado um *cutoff* de 94%, em que os isolados que apresentassem maior ou igual percentagem seriam considerados como sendo o mesmo clone [3]. Cinco agregados foram arrolados para estudo, apenas os resultados de dois são demonstrados neste poster, devido às dimensões dos dendrogramas resultantes.

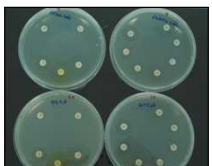
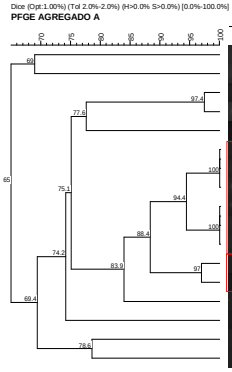
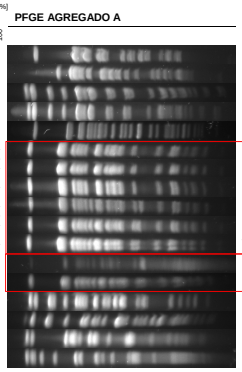


Figura 1 – Seleção de isolados com base na semelhança do seu fenótipo de resistência e perfil com múltiplas resistências.

Resultados



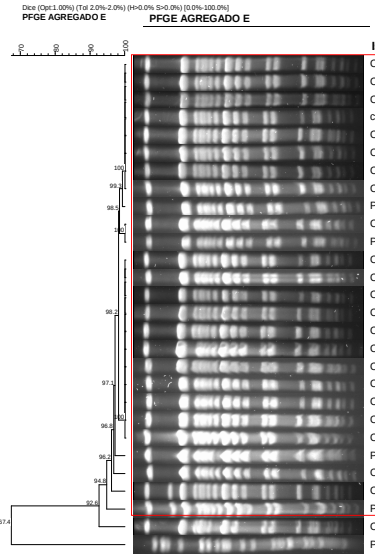


Isolado	Fenótipo Resistência	Filogrupo
Proprietária - fezes	C CIP K NA SXT TE	B1
Gata - fezes	K NA S TE	B1
Proprietário - fezes	AMP ATM CAZ C CTX KF TE S STX TOB	B1
Proprietário - fezes	AMP ATM CAZ C CTX CN K KF S SXT TE TOB	B1
Cadela - urina	AMP ATM CAZ C CIP KF NA S TE	B1
Porta frigorífico	AMP ATM C CIP KF NA S TE	A
Cadela - boca	AMP ATM C CAZ CIP KF NA S TE	A
Chão marquise	AMP ATM C CAZ CIP KF NA S TE	A
Cadela - boca	AMP ATM C CIP CN K KF NA S SXT TE TOB	A
Cadela - fezes	AMP ATM C CAZ CIP CN K KF NA S SXT TE TOB	A
Taça comida	AK AMC AMP ATM C CIP CN K KF NA TE S SXT TOB	A
Proprietário - fezes	AMP ATM C CAZ CTX K KF S SXT TE	A
Cadela - fezes	AMP C CAZ CIP CN KF K NA TE S SXT TOB	A
Proprietária - fezes	AMP S SXT TE	B1
Proprietária -fezes	NA S SXT TE	A
Bébe - fezes	AMP NA S SXT	D
Porta frigorífico	AMC AMP ATM C CAZ CIP KF NA S TE	D

Figura 2 – Dendrograma Agregado A obtido por PFGE - Este agregado é constituído por uma cadela com uma doença crónica de pele para a qual foi tratada com várias terapias antimicrobianas assim como o seu proprietário, (e.g. amoxicilina - ácido clavulânico, ciprofloxacina). Do "agregado familiar" fazia também parte um neto ainda bebé e uma gata de interior, saudáveis e sem terapias anteriores. Isolaram-se estirpes de *E.coli* com fenótipos de resistência similar procedendo-se a estudos de proximidade clonal por genotipagem (PFGE). A vermelho estão realçados alguns conjuntos de isolados com similaridades superiores a 94%.

Disseminação de um clone de *E.coli* com múltiplas resistências da boca e fezes da cadela para o ambiente doméstico.

Partilha de um clone de *E.coli* multirresistente entre a cadela e o seu proprietário



Isolado	Fenótipo de resistência	Filogrupo
Cadela - coleira	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cadela - taça comida	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cadela - brinquedo	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
cadela - fezes	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cadela - boca	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cadela - pelo	AMP, FOX, IPM, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - fezes 1	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Corrimão 2	AMP, FOX, CIP, CN, TE, CTX, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Proprietário - mãos	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Chão	AMP, FOX, CIP, CN, TE, CTX, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Proprietário - fezes 1	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - fezes 2	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cães - cama	AMP, FOX, CIP, CN, TE, CTX, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - boca	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - pelo 1	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - pele	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - taça comida 1	AMP, FOX, CIP, CN, TE, CAZ, KF, S, NA, C, TOB, SXT, K	A
Cão B - fezes	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão B - coleira 1	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão B - coleira 2	AMP, FOX, CIP, CN, TE, CAZ, KF, S, NA, C, TOB, SXT, K	A
Cão B - taça comida	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Corrimão 1	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Porta frigorífico	AMP, FOX, CIP, CN, TE, CTX, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão B - boca	AMP, FOX, IPM, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Cão A - pelo 2	AMP, FOX, CIP, CN, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Proprietário - fezes 2	AMP, FOX, CIP, CN, CAZ, AMC, KF, AK, S, NA, C, TOB, SXT, K	A
Cão A - taça comida 2	AMP, FOX, CIP, CN, TE, CAZ, AMC, KF, S, NA, C, TOB, SXT, K	A
Proprietária - fezes	AMP, S	B2

Figura 3 – Dendrograma Agregado E obtido por PFGE - Este agregado é constituído por um cão (cão A) que apresenta otites alérgicas e purulentas, motivo pelo qual foi sujeito a várias terapias antimicrobianas. Do mesmo agregado faziam parte outro cão (cão B) e uma cadela, saudáveis, assim como os seus proprietários. Selecionaram-se estirpes de *E.coli* com um perfil de resistência similar e aparentemente disseminado, procedendo-se a estudo genético por PFGE. Isolaram-se também quatro conjuntos de *Enterococcus faecalis*, organizados com base em quatro fenótipos de resistências que se encontravam disseminados, que foram analisados por ERIC PCR (dendrograma não apresentado, representativo das mesmas conclusões) .

Disseminação de um clone de *E. coli* entre diferentes membros do agregado e ambiente doméstico.

Um clone de *E. coli* colonizou várias partes do corpo do animal, além do trato intestinal.

Conclusão

Detetou-se transferência de clones bacterianos com múltiplas resistências entre os coabitantes humanos e animais do mesmo agregado familiar, com disseminação para superfícies e objetos domésticos

É necessário:
- Fazer um uso prudente dos antimicrobianos - impacto na qualidade de vida dos animais e também na saúde humana.

- maior vigilância nas formas de interação com os animais e a adoção de medidas higiénicas cautelares após essa interação.

Abreviaturas: AK - Amicacina , AMC - Amoxicilina/ácido clavulânico , AMP - Ampicilina, ATM - Aztreonamo, AZM - Azitromicina, C - Cloranfenicol , CAZ - Ceftazidima , CIP - Ciprofloxacina , CN - Gentamicina , CTX - Cefotaxima , E - Eritromicina , F – Nitrofurantoína, FOX - Cefoxitina , IPM - Imipenem , K - Kanamicina , KF - Cefalotina , NA - Ácido nalidíxico , S - Estreptomicina , SXT - Sulfametoxazol/trimetoprima, TE - Tetraciclina , TOB - Tobramicina , ITU – Infeção do trato urinário.

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Comunicação Poster

IN-HOME AND THROUGH-HOME TRANSMISSION OF ANTIMICROBIAL RESISTANCE BETWEEN HUMAN AND PETS

Leite-Martins, L., Beça, N., Lopes, E., Frias, C., Matos, A., Martins da Costa, P.

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In-home and through-home transmission of antimicrobial resistance between human and pets

L. Leite-Martins[1,2], N. Beça[2], E. Lopes[2], C. Frias[1], A. de Matos[1] ,

P. Martins da Costa[2]

[1]UPVET Clínica Veterinária da Universidade do Porto, Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal

[2]Laboratório de Microbiologia, Departamento de Produção Aquática, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal

In the last decade, there has been an increasing awareness of the potential problems that selection of antimicrobial resistant bacteria among companion animals may cause on human health, due to the increasing use of the same antimicrobial substances in both species and to the close contact between pets and their human co-habitants.

The aims of the present study were to characterize the antimicrobial resistance profiles among *Escherichia coli* and enterococci strains isolated from cohabitant pets and humans, evaluating the concurrent colonization of pets, owners and home surfaces by bacteria carrying the same antimicrobial resistance genes.

Three domestic aggregates (A, B and C) were selected from the universe of clients of UPVET (Porto University Veterinary Clinic, Portugal). After a study explanation, clients signed the agreement and ethical documents and answered to a complete questionnaire. Samples were delivered and/or collected as soon as possible (faeces, urine, oral swabs, home surfaces swabs and hands swabs) and *E. coli* and enterococci were isolated. Susceptibility to antimicrobial agents was tested using the disk diffusion method. The identification of antimicrobial resistance genes was made through polymerase chain reaction amplification using previously described probes.

From the aggregate A, a total of 124 *E. coli* isolates were recovered displaying 24 different resistance patterns with a remarkable percentage of multi-resistant ones

(46% displayed simultaneous resistance to at least nine different antimicrobials). Strains displaying the same resistance patterns were isolated from the dog's urine and mouth, laundry floor, refrigerator door and dog's food bowl. Other multi-resistant phenotypes and resistance genes were found repeatedly in different inhabitants and surfaces of the house. From the domestic aggregate B, the same resistance phenotype was found among enterococci isolated from the cat faeces and the two home inhabitants (female owner and her daughter). Finally, from the domestic aggregate C, enterococci isolated from faeces and oral secretions of the dog and the hands of both owners exhibited the same resistance pattern encompassing simultaneous resistance against eight antimicrobial drugs. In order to analyse phylogenetic and epidemiological relations between the *E. coli* strains from domestic aggregate A, a polymerase chain reaction (PCR) of enterobacterial repetitive intergenic consensus sequences (ERIC) was performed. Through the dendrogram overview, the same isolates (100% similarity) existed in the dog mouth, it's food bowl, the kitchen floor and the refrigerator door. With 93% similarity the above strains plus the ones from the dog's faeces and urine, the cat faeces and its owner faeces, can be said to belong to the same clone.

Direct, close contact between all the cohabitants and the touch of contaminated household surfaces and objects could be an explanation for these observations. These findings raise questions regarding the potential contribution of shared household surfaces in antimicrobial resistance transfer between animal and human cohabitants. Finally it was established that a pet can orally transport *E. coli* strains with the same antimicrobial resistance profile of their faecal and urinary strains, which could be explained by some frequent behaviour of dogs such as rolling on faeces, grooming and perigenital licking. The presence of those resistant strains in the dog's mouth is likely to have played a key role in their spread. Although resistance patterns are not static, the genotypic and phenotypic correspondences demonstrated in this applied study suggest interspecies transmission. Furthermore, the finding that almost all of the resistance genes were also present among strains isolated from the household environment, could be indicative of an in-home and through-home transmission.

Keywords: antimicrobial; resistance; pets; home; surfaces

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In-home and through-home transmission of antimicrobial resistance between human and pets

L. Leite-Martins[1,2], N. Beça[2], E. Lopes[2], C. Frias[1], A. de Matos[1] and P. Martins da Costa[2]

[1]UPVET Clínica Veterinária da Universidade do Porto, Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal
[2]Laboratório de Microbiologia, Departamento de Produção Aquática, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal

Introduction

Antimicrobial resistance is a major public-health problem worldwide [2,3]. In the last decade, there has been an increasing awareness of the potential problems that the selection of antimicrobial resistant bacteria among companion animals may cause on human health due to the increasing use of the same antimicrobial substances in both species and to the close contact between pets and their human co-habitants [2].

The aims of the present study were to characterize the antimicrobial resistance profiles among *Escherichia coli* and enterococci strains isolated from cohabitant pets and humans, evaluating the concurrent colonization of pets, owners and home surfaces by bacteria carrying the same antimicrobial resistance genes, assessing the possible contribution of household surfaces to the in-home and through-home spread of antimicrobial resistance.

Materials and Methods

Three domestic aggregates (A, B and C) were selected from the universe of clients of UPVET (Porto University Veterinary Clinic, Portugal). After a study explanation, clients signed the agreement and ethical documents and answered to a complete questionnaire about environment, human and veterinary medical records with antibiotic usage by themselves, family members and their pets. Samples were delivered and/or collected as soon as possible (faeces, urine, oral swabs, home surfaces swabs and hands swabs) and *E. coli* and enterococci were isolated according to protocols followed by others [1,3,4]. Susceptibility to antimicrobial agents was tested using the disk diffusion method and clinical and laboratory standards guidelines were followed (CLSI, 2007). The identification of antimicrobial resistance genes was made through polymerase chain reaction amplification using previously described probes [1,3,4].

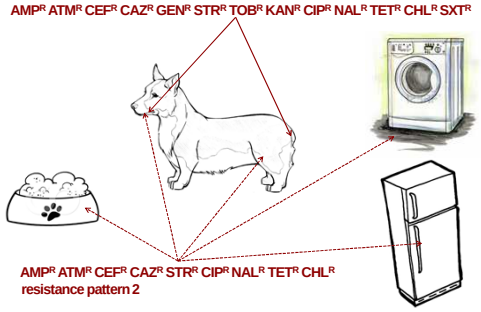
Results

Domestic aggregate A

Escherichia coli

resistance pattern 1

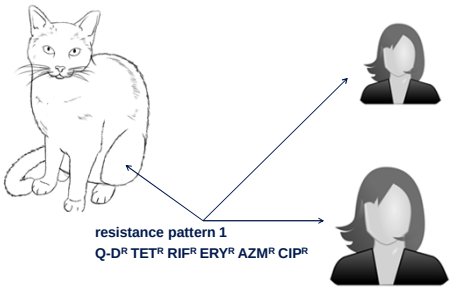
AMP^R ATM^R CEF^R CAZ^R GEN^R STR^R TOB^R KAN^R CIP^R NAL^R TET^R CHL^R SXT^R



AMP^R ATM^R CEF^R CAZ^R STR^R CIP^R NAL^R TET^R CHL^R
resistance pattern 2

Domestic aggregate B

enterococci



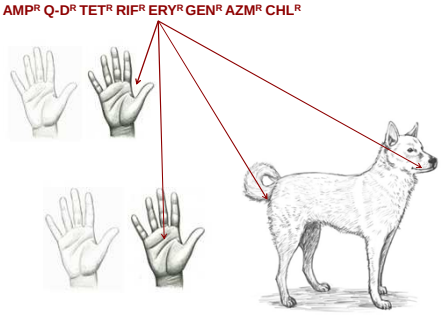
resistance pattern 1
$Q\text{-}D^R$ TET^R RIF^R ERY^R AZM^R CIP^R

Domestic aggregate C

enterococci

resistance pattern 1

AMP^R $Q\text{-}D^R$ TET^R RIF^R ERY^R GEN^R AZM^R CHL^R



From aggregate A, one multidrug-resistant strain isolated from the dog's faeces was also found in it's oral cavity; the isolate from the dog's urine was found in it's mouth, laundry floor, refrigerator door and dog's food bowl. Other multi-resistant phenotypes and resistance genes were found repeatedly in different inhabitants and surfaces of the house. From the domestic aggregate B, the same resistance phenotype was found among enterococci isolated from the cat faeces and from two of the home inhabitants. From the domestic aggregate C, enterococci isolated from faeces and oral secretions of the dog and the hands of both owners exhibited the same resistance pattern encompassing simultaneous resistance against eight antimicrobial drugs.

Discussion

Direct, close contact between all the cohabitants and the touch of contaminated household surfaces and objects could be an explanation for the above observations. These findings raise questions regarding the potential contribution of shared household surfaces in antimicrobial resistance transfer between animal and human cohabitants. Finally it was established that a pet can orally transport *E. coli* strains with the same antimicrobial resistance profile of their faecal and urinary strains, which could be explained by some frequent behaviour of dogs such as rolling on faeces, coprophagy, grooming and perigenital licking. The presence of those resistant strains in the dog's mouth is likely to have played a key role in their spread. Although resistance patterns are not static, the genotypic and phenotypic correspondences demonstrated in this applied study suggest interspecies transmission. Furthermore, the finding that almost all of the resistance genes were also present among strains isolated from the household environment, could be indicative of an in-home and through-home transmission.

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Comunicação Poster

CULTURE MEDIA ISOLATION OF *STAPHYLOCOCCUS PSEUDOINTERMEDIUS* AND *STAPHYLOCOCCUS* SPP. COAGULASE POSITIVE PREVALENCE IN DOMESTIC ANIMALS, VETERINARY PRACTITIONERS, VETERINARY AUXILIARY WORKERS AND ENVIRONMENT OF A VETERINARY HOSPITAL

Beça, N.M., Simões, R.L., Santos, J.C., Lopes, E., Leite-Martins, L., Matos, A., Martins da Costa, P.

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Culture media for isolation of *Staphylococcus pseudointermedius* and *Staphylococcus* spp coagulase positive, prevalence in domestic animals, veterinary practitioners, veterinary auxiliary workers and environment of a Veterinary Hospital

N.M. Beça[1], R. L. Simões [1], J. C. Santos [2], E. Lopes[1], L. Leite-Martins[2], A. Matos [2] P. Martins da Costa[1]

[1] Laboratório de Microbiologia-Tecnologia Alimentar- Inspeção Sanitária, Departamento de Produção aquática, Instituto de Ciências Biomédicas Abel Salazar ,Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal

[2]UPVET Clínica Veterinária da Universidade do Porto, Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal

Abstract

Staphylococcus aureus and *Staphylococcus pseudointermedius* are well recognized as potential pathogens in both animal and human medicine. In the present study, oral, nasal and skin swabs were collected from 21 dogs and 2 cats attended in a Veterinary Hospital in Porto, Portugal, from veterinary practitioners and auxiliary workers (hands and nose), and from nine different contact surfaces used by veterinary practitioners, auxiliary workers and animals.

Swabs were cultured in Baird Parker – Rabbit Plasma fibrinogen (Biokar) and incubated at 37º C for 36 hours. Subsequently all coagulase-positive *Staphylococcus* were subcultured onto Chromagar STAPHaureus (CHROMAGAR) and then screened for antimicrobial susceptibility. Polymerase Chain Reaction was performed with primers for *S. aureus* (*au-F3*, and *au-nucR*) and *S. pseudointermedius* (*pse-F2*, *pse-R5*,) in order to identify the *Staphylococcus* species.

All colonies exhibiting typical *S. aureus* morphology (mauve colour) and all purple-blue coloured colonies were identified as *S. aureus* and *S. pseudointermedius*, respectively. This procedure has proven to be reliable for *S. pseudointermedius* isolation, being an alternative to the laborious and time consuming biochemical tests.

Among the tested animals, 65.2% (n=15) carried coagulase-positive *Staphylococcus*: 30.4% (n=7) *S. aureus* and 52.2% (n=12) *S. pseudointermedius*. Two dogs (8.7%) carried methicilin resistant *Staphylococcus aureus* (MRSA) and four (17.4%) dogs were colonized with methicilin resistant *Staphylococcus pseudointermedius* (MRSP). Antimicrobial resistances to amoxicillin, thrimethropim-sulphamethoxazole and lomefloxacin were the most common in MRSP carriers. Four animals carried both *S. aureus* and *S. pseudointermedius* from the same swabs. In two animals, MRSP isolates presenting more than one antimicrobial resistance profile were found in the isolation place. Oral and nasal mucosae were the animal locations where more *S. aureus* bacteria were isolated while *S. pseudointermedius* were isolated mostly in oral mucosae and skin.

Among the environment swabs, *S. pseudointermedius* was isolated from the floor of the Hospital recovery area and the computer keyboards, both isolates being MRSP. *S. aureus* was found only in computers keyboards.

Regarding the nine veterinary practitioners and auxiliary workers tested, in all hand samples and in 22.2% of the nasal swabs, *Staphylococcus* displaying coagulase positive activity were isolated. Hand isolates consisted of *S. aureus* in 88.9% (n=8) and *S. pseudointermedius* in 55.6% (n=5), one of which was MRSP. Only 22.2% (n=2) presented *S. aureus* in nose samples and none *S. pseudointermedius* was isolated.

S. aureus isolated from computer keyboard and veterinary practitioners displayed the same resistance pattern.

This last fact alerts to the necessity of good hygiene practices such as hand washing, aseptic practices and good surface disinfection during all processes of animal management.

Keywords: *Staphylococcus aureus*, *Staphylococcus pseudointermedius*, antibiotic resistance; veterinary practice

Culture media for isolation of *Staphylococcus pseudointermedius* and *Staphylococcus* spp coagulase positive -prevalence in domestic animals, veterinary practitioners, veterinary auxiliary workers and environment of a Veterinary Hospital

NunoM. Beça[1], R. L. Simões [1], J. C. Santos [2], E. Lopes[1], L. Leite-Martins[2], A. Matos [2], P. Martins da Costa[1]

[1] Laboratório de Microbiologia-Tecnologia Alimentar- Inspeção Sanitária, Departamento de Produção aquática, Instituto de Ciências Biomédicas Abel Salazar ,Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal
[2]UPVET Clínica Veterinária da Universidade do Porto, Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal

INTRODUCTION

Staphylococcus aureus and Staphylococcus pseudointermedius are two coagulase positive Staphylococcus with high relevance in Veterinary Medicine (Weese et al,2010). S. pseudointermedius is a comensal bacteria in small animal which can be associated to Immunocompromised cases, atopic allergy cases or surgical procedures (Bannoehr and Guardabassi, 2012).

MATERIAL AND METHODS

Oral, nasal and skin swabs were collected from 21 dogs and 2 cats attended in a Veterinary Hospital in Porto, Portugal, from veterinary practitioners and auxiliary workers (hands and nose), and from nine different contact surfaces used by veterinary practitioners, auxiliary workers and animals. Swabs were cultured in Baird Parker – Rabbit Plasma fibrinogen (Biokar) and incubated at 37º C for 24 hours. Plaques observation for coagulase activity were done at 24 and then let at environmental temperature between 22-24ºC for further coagulase activity observation.Subsequently all coagulase-positive *Staphylococcus* were subcultured onto Chromagar STAPHaureus (CHROMAGAR) and then screened for antimicrobial susceptibility test. Polymerase Chain Reaction was performed with primers for *S. aureus* (*au-F3*, and *au-nucR*) and *S. pseudointermedius* (*pse-F2*, *pse-R5*,) in order to identify the *Staphylococcus* species. (Sasaki et al. 2010).

RESULTS

Table 1. Prevalence by phenotipe of the isolated coagulase positive *Staphylococcus* on the 23 tested animals

Phenotipe	Number (%)
MSSA	5 (21.7 %)
MRSA	2 (8.7 %)
MSSP	8 (34.8 %)
MRSP	4 (17.4 %)

***S. pseudointermedius* were isolated the most at oral mucosae and skin**
***S. aureus* were isolated the most at oral mucosae**

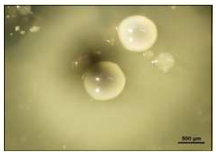


Figure 1. Macroscopique picture of *S. pseudointeremedius* colonies. *S. pseudointermedius* coagulase positive activity started 4 hours after the plaque was left at environmental temperature (22-24 ºC)

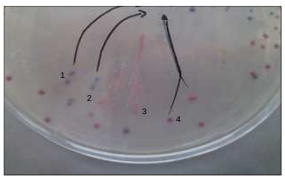


Figure 2. CHROMagar STAPhaureus colony colours: ***S. pseuintermedius*** purple (1) and blue (2) colors; ***S. aureus*** mauve (3) and dark mauve (4) colours

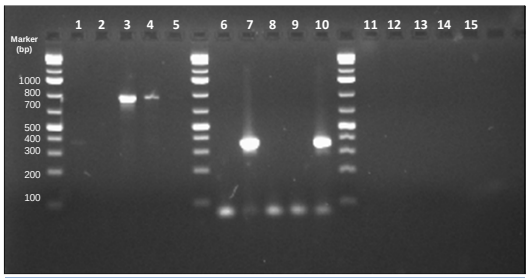
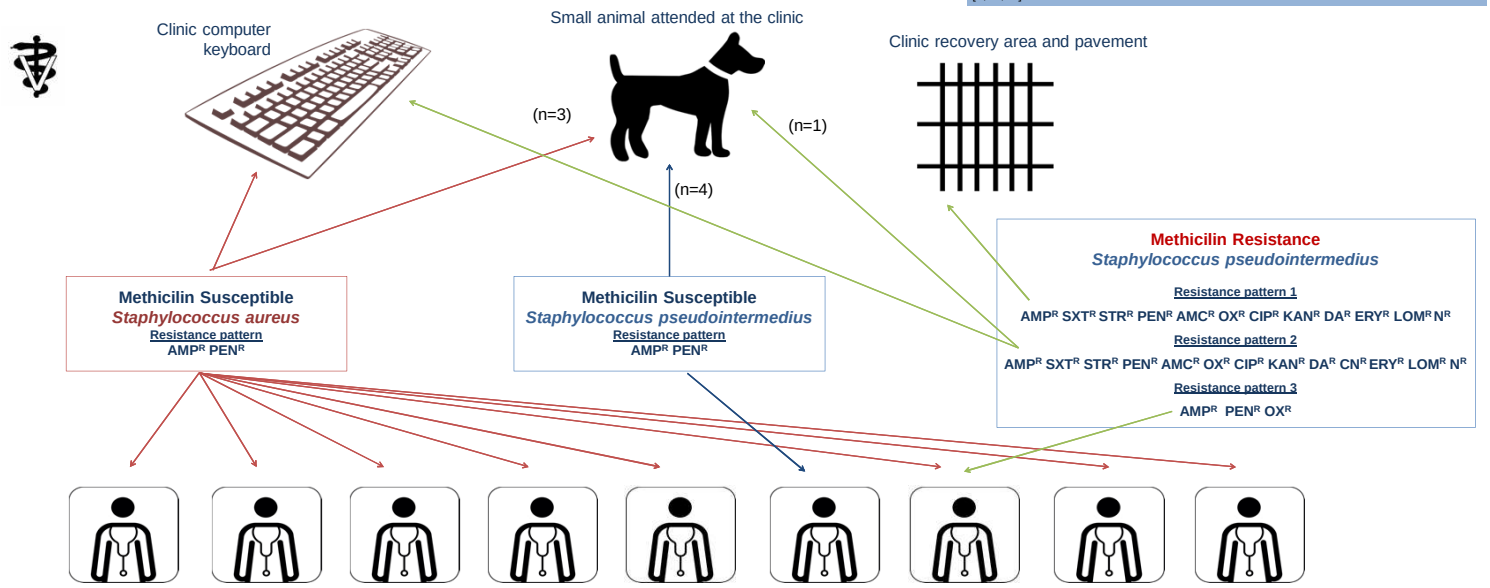


Figure 3. Eletrophoresis in 1.5 % gel agarose after PCR

1-5 primers pse-F2 e pse-R5 – *S. Pseudointermedius* // 6-10 primers au-F3 e au-nucR – *S. Aureus* // 11-15 – without adding primers [1,6,11] **Animal ID6 oral sample DNA(not relevant for this case) //** [2,7,12] **Animal ID22 oral sample DNA ,mauve colour in CHROMagar //** [3,8,13] **Animal ID22 oral sample DNA ,purple colour in CHROMagar // 4,9,14- Animal ID22 oral sample DNA blue colour in CHROMagar //** [5,10,15]**- *S. aureus* ATCC 25 293 DNA**



CONCLUSIONS

The combined use of BHI supplemented with Tween 80 and further culture on Baird Parker- RPF and CHROMagar Staphaureus confirmation appears to be a very reliable method for *Staphylococcus pseudointermedius* isolation.
Antimicrobial resistance pattern for each animal attending the small animal clinic might seem necessity in every pathological cases, in order to perform an animal effective treatment. Mutual information exchange between Microbiology Laboratories and Small Animal Clinic/ Hospitals can not only provide an effective animal treatment but also avoid empirical antibiotic treatments and public health threats.
Good hygiene practices such as hand washing, aseptic practices and good surface disinfection are key factors during all animal management processes.